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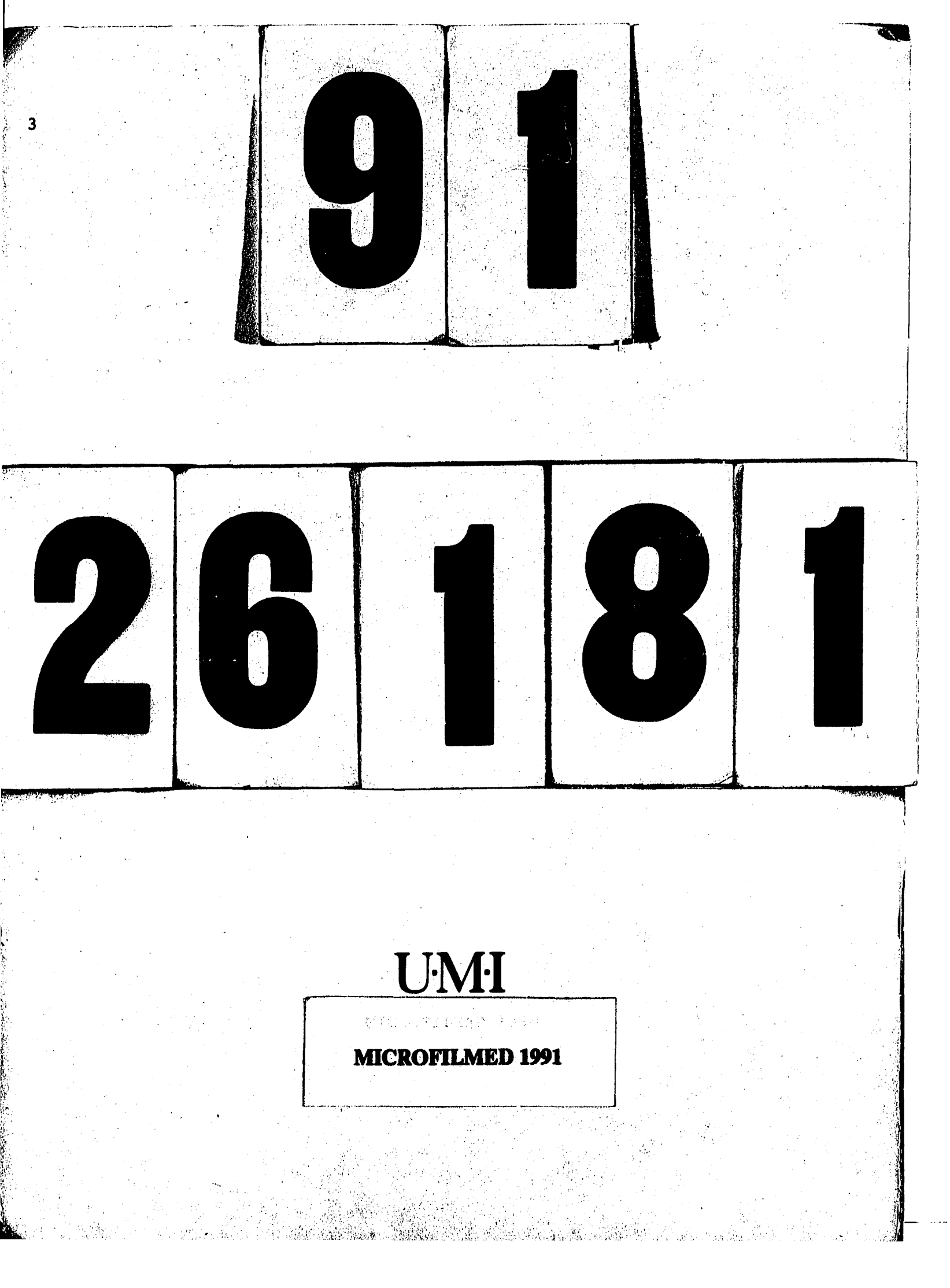
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Purification and properties of the malic enzyme and the malate dehydrogenases from the gill tissue of the ribbed mussel (Modiolus demissus)

by

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TABLE OF CONTENTS

INTRODUCTION

The ribbed mussel (Modiolus demissus) lives high in the intertidal zone of estuarine environments and is therefore faced with potential aerial exposure as well as shifts in the osmolality of its bathing medium. The measurable changes in the pattern of accumulation of various metabolites indicate that it adjusts its metabolism in response to these environmental stresses (Ho and Zubkoff, 1982, 1983; Baginski and Pierce, 1977). As glycolysis and possibly amino acid metabolism contribute to the metabolites synthesized (Collicut and Hochachka, 1977; deZwaan, 1977), enzymes of intermediary metabolism in these organisms might possess interesting regulatory properties. The purification and properties of the cytosolic malate dehydrogenase (cMDH), the mitochondrial malate dehydrogenase (mMDH), and the strictly mitochondrial malic enzyme (ME) from the gill tissue of M. demissus (Paynter et al., 1985a) are considered to assess their possible role in the synthesis of metabolites accumulating under environmental stress.

Responses to Limiting Oxygen Availability (Anoxia)

The ribbed mussel is found at the highest point in the intertidal zone of any mollusc. Members of several populations spend more time exposed to air than covered with water (Lent, 1969). Many intertidal bivalves close their shells when faced with aerial exposure. Valve closure apparently reduces energy demand as feeding, locomotion, and gill movements stop (Gäde, 1983a). The ribbed mussel and the closely related blue mussel (Mytilus edulis) gape air periodically when aerially exposed (Lent, 1968; Schick et al., 1986)

which permits oxygen uptake (Booth and Mangum, 1978). Correlated with air gaping are periods of increased heat production (Pomatmat, 1983; Schick et al., 1986) indicating increased metabolic activity (Schick et al., 1983).

Despite their air gaping behavior, ribbed mussels like other molluscs and some parasitic helminths synthesize and accumulate several endproducts in response to anaerobic stress. Succinate and alanine accumulate rapidly in the tissue of the ribbed mussel in response to several hours to days of exposure to air. Longer term exposure causes a reduction in the rate of succinate production, while alanine levels continue to rise. Several days of exposure also result in large concentrations of propionate, and small quanitities of lactate, acetate. butyrate and isobutyrate (Ho and Zubkoff, 1982, 1983). The responses of the blue mussel are similar: succinate, alanine, and propionate are the major anaerobic endproducts (Kluytmans et al., 1977). Smaller amounts of butyrate and isovalerate also appear as anaerobic endproducts in the blue mussel (Kluytmans et al., 1975, 1978).

The pathways for the synthesis of some of these compounds have been described in several diverse species. Succinate, an anaerobic endproduct in many diverse phyla with anaerobic members, is produced by at least two different pathways. The accumulation of about half of the label from labeled glutamate supplied to isolated oyster ventricles into succinate is evidence for the anaerobic formation of succinate from the forward operation of Krebs cycle activities from at least the level of alpha-ketoglutarate dehydrogenase (Collicut and Hochachka, 1977). In addition, succinate is formed by an electron transport chain-linked fumarate reductase (Tubifex sp.: Schöttler,

1977a, 1977b; M. edulis: Holwerda and deZwaan, 1980; Hymenolepis diminuta: McKelvey and Fioravanti, 1985). The association of this activity with the electron transport chain results in the production of ATP concurrent with succinate (Kröger, 1978). Although the fumarate reductase catalyzes essentially the reverse reaction of the succinate dehydrogenase, several lines of evidence suggest that these activities reside in distinct proteins (Hederstedt and Rutberg, 1981; Saz, 1981).

During the initial adjustment to anaerobic stress, the large free aspartate pools found in members of most mollusc species serve as precursor for the synthesis of succinate (Gäde, 1983b; Livingstone, 1982; Schöttler et al., 1983). For example, in isolated oyster ventricles incubated anaerobically, the majority (50%) of supplied radiolabeled aspartate distributes into succinate (Collicut and Hochachka, 1977). Additional evidence that aspartate serves as succinate precursor in the oyster ventricle is the observation that treatment with the transaminase inhibitor aminooxyacetic acid significantly inhibits utilization of aspartate while inhibiting the accumulation of succinate as well as alanine (Foreman and Ellington, 1983). Transamination is also necessary for the synthesis of succinate in the adductor muscle of the blue mussel (deZwaan et al., 1982; deZwaan et al., 1983a).

While aspartate is the main succinate precursor during initial anaerobic adjustment (Gäde and Meinardus, 1981; Gäde, 1983b), prolonged exposure causes a metabolic alteration in which glucose becomes the main succinate precursor at the expense of tissue glycogen content (Gäde, 1975; deZwaan et al., 1982). Because label from $^{14}CO_2$ accumulates in succinate in tissue of the

ribbed mussel (Baginski and Pierce, 1978), carboxylation of a glycolytic three carbon intermediate is indicated. The active PEPCK found in most molluscs is the most likely site for this carboxylation (Hochachka and Mustafa, 1972; Harlocker et al., 1991). Carboxylation of PEP is also observed in the anaerobic synthesis of succinate in many parasitic helminths (Saz, 1981). Consistently, the PEPCK inhibitor 3-mercaptopicolinic acid limits the accumulation of succinate in most of the tissues of the blue mussel (deZwaan et al., 1983a). The effect of 3-mercaptopicolinic acid on the isolated adductor muscle of the blue mussel is dependent on the season at which the animals are collected with the PEPCK reaction more important in summer than in winter in the formation of succinate (deZwaan et al., 1983a). That little glucose-derived label is detected in succinate in anaerobic oyster ventricles is explained by this tissue's containing low levels of the PEPCK activity (Collicut and Hochachka, 1977).

The pathways responsible for the synthesis of the propionate and acetate which accumulate in response to anaerobic exposure (Ho and Zubkoff, 1983) have been described for some parasitic helminths but have not been elucidated in molluscs. The formation of propionate and acetate in molluscs does have in common with their formation in parasitic helminths a link to mitochondrial malate metabolism (deZwaan et al., 1981; Saz, 1981). Succinate (derived from malate) is the precursor of propionate formation in the parasitic helminths Spirometra mansonoides (Tkachuck et al., 1977; Pietrzak and Saz, 1981) and Fasciola hepatica (Pietrzak and Saz, 1981) in a series of reactions which yield ATP. Acetate production in Fasciola hepatica also yields ATP (vanVugt et al., 1979). A thiol transferase activity is implicated in the

formation of propionate from succinate as acyl CoA derivatives stimulate the propionate forming reactions. Because acetyl CoA stimulates the synthesis of propionate from succinate much more than propionyl CoA, acetyl CoA might be the actual CoA donor for formation of succinyl CoA under physiological conditions (Pietrzak and Saz, 1981). Acetyl CoA acting as the substrate for an acyl transferase in the synthesis of succinyl CoA might also account for acetate's accumulating under anaerobic conditions in both molluscs and parasitic helminths. In molluscs, the majority of propionate might derive from the succinyl CoA product of the alpha-ketoglutarate dehydrogenase reaction, as arsenite (a blocker of the lipoamide cofactor linked dehydrogenases alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase) significantly inhibits the anaerobic production of propionate from malate (deZwaan et al., 1981). However, arsenite could also exert this effect by inhibiting the production of acetyl CoA by the pyruvate dehydrogenase reaction. Propionate production might then be limited by the limiting concentration of substrate (acetyl CoA) for the thiol transferase reaction. Because label from 2,3-^{14}C succinate appears in propionate during anaerobic metabolism by the blue mussel (Wijsman et al., 1977), the production succinyl CoA via an acetyl CoA transthiolation may be indicated. Some reversibility of these reactions is indicated, as labeled propionate is metabolized to succinate by mollusc tissue (Hammen and Wilbur, 1959).

Although succinate accumulates as an anaerobic endproduct in molluscs, it remains metabolically active. Succinate derived label distributes into a number of compounds, especially malate, aspartate, glutamate, and alanine, providing evidence that the remainder of the Krebs cycle is functional during

anaerobiosis (Wijsman et al., 1977). That succinate formed during anaerobic conditions is rapidly metabolized when the organisms are returned to aerobic conditions is indicated by a sodium malonate-inhibiting increase in O_2 consumption (Malango and Ayello, 1972). One of succinate's metabolic fates is contribution to the recovery of aspartate pools (Gäde and Meinhardus, 1981). While a similar increase in O_2 consumption was not observed when tissue of the blue mussel was returned to aerobic conditions, recovery from anaerobiosis produces some increase in metabolic rate as assessed from increased heat production and increase in CO_2 production (Fame and Knudsen, 1983).

Several possible regulators of the anaerobic production of succinate, and therefore probably for acetate and propionate as well, have been proposed. Anaerobic incubation produces a measurable drop in the pH of the pallial fluid (Wijsman, 1975) and hemolymph (Booth et al., 1984) of the blue mussel, which has been attributed to increased P_{CO2} in the hemolymph (Booth et al., 1984; deZwaan et al., 1983). Actual tissue pH decreases were observed with ^{31}P n.m.r. in the anaerobic catch muscles of the blue mussel and the ribbed mussel (Ellington, 1983a), in the ship worm (Tapes watlingii: Barrow et al., 1980), and in the ventricles of the whelk (Busycon contrarium: Ellington, 1983b), and by chemical means in the blue mussel (Walsh et al., 1984). Hochachka et al. (1973) and Hochachka and Somero (1973) predicted this anoxic decrease in pH would be an important regulator of the enzymes PEPCK and pyruate kinase. According to their scheme, the lower pH favors the PEPCK reaction, leading to the observed fixation of carbon for the synthesis of succinate. Recently, it has been shown that anoxia produces an

intracellular signal for the phosphorylation of pyruvate kinase which changes the regulatory properties of this enzyme (Michaelidis and Storey, 1990). Specifically, the $S_{0.5}$ and cooperativity for PEP increase, the K_a for fructose-1,6-diphosphate increases, and the I_{50} for alanine decreases. The net result of decreasing pH activating PEPCK and phosphorylation inactivating PK is the enhanced carboxylation of PEP destined for succinate synthesis.

The majority of alanine which accumulates in molluscs during anoxia is derived from glucose (Collicut and Hochachka, 1977); however inhibition of glycolysis with iodoacetate does not totally inhibit alanine synthesis (deZwaan et al., 1982). Therefore, alanine may derive from another precursor in molluscs. As label from aspartate appears in alanine (about 11% of total) and the alanine derivative alanopine (about 16% of total), aspartate also serves as a precursor (Hochachka and Collicut, 1977; Gäde and Ellington, 1983). Consistently, the amount of aspartate lost during anaerobic incubation cannot be accounted for by the amount of succinate formed (cockle: Gäde, 1983b; blue mussel: deZwaan et al., 1983a). The simplest metabolic route for the synthesis of alanine from aspartate requires a decarboxylation step and malic enzyme has been suggested as the activity responsible. However, when 4-^{13}C aspartate or 2-^{13}C aspartate was supplied to isolated whelk ventricles, no label was detectable in alanine (Graham and Ellington, 1985). Therefore, in order for asparatate to serve as a precursor for alanine, a more complex metabolism may be required.

Alanine synthesized during anaerobic stress, like succinate, remains metabolically active. About 30% of the label from radiolabeled alanine

supplied to the isolated anaerobic oyster ventricle distributes into pyruvate, indicating a transamination reaction. The remaining 70% distributes into the opine alanopine (Collicut and Hochachka, 1977; Fields, 1983). Alanopine is formed by the redox reestablishing condensation of alanine and pyruvate (Fields, 1983). Alanopine is one of several opines formed by mollusc tissue (Livingstone et al., 1981; Gäde et al., 1978; deZwaan and Zurburg, 1981). Other opines include strombine (the condensation product of glycine and pyruvate), and octopine (the condensation product of arginine and pyruvate). Because 30% of the label of supplied radiolabeled glucose was found in alanopine in the isolated anaerobic oyster ventricle, opine production depends on the glycolytic production of pyruvate (Collicut and Hochachka, 1977).

Alanine synthesis in anoxic adjustment apparently requires a nitrogen fixing mechanism because the amount of alanine formed is greater than the amount of aspartate consumed (deZwaan, 1983). Ammonia is excreted during anaerobic exposure in a time dependent manner (deVooys and deZwaan, 1978); therefore amino nitrogens are possibly released from protein catabolism. Molluscs possess several amino acid metabolizing systems, including a particulate associated L-amino acid oxidase (Burcham et al., 1980), a low activity glutamate dehydrogenase (Reiss et al., 1977), and several a-KG/ GLU linked transaminases (Paynter et al., 1984a, 1984b; Bishop et al., 1981). The formation of alanine by pathways dependent on glutamate/alpha-ketoglutarate dependent transaminases in a polychaete worm is indicated by the transfer of ^{15}N from glutamate to alanine (Felbeck, 1980). The importance of these transaminases in the formation of alanine in mollusc tissue is

indicated by the observation that aminooxyacetate reduces the amount of alanine formed (Foreman and Ellington, 1983: Bishop et al., 1981).

While alanine, succinate, propionate and opines are the major endproducts synthesized by molluscs in response to anaerobic stress, smaller amounts of other anaerobic endproducts are also synthesized. Many of these compounds are formed by other invertebrates with anaerobic capacity. These include D-lactate which is probably produced by D-lactate dehydrogenase in the cockle (Gäde, 1983a) and polychaete worm (Schöttler and Weinhauser, 1981), but which could be produced from methylglyoxal via a glutathione dependent pathway as is observed in Leishmania sp. (Darling et al., 1989; Darling and Blum, 1988).

Small quanitities of branched chain fatty acids are formed by molluscs in response to anaerobic stress by pathways which, like those leading to the formation of acetate and propionate, have not been elucidated. Larger quantities of branched chain fatty acids are formed by the parasitic roundworm Ascaris suum by the condensation of acyl CoA derivatives (either two molecules of propionyl CoA for the formation of 2-methylvalerate or one molecule of acetyl CoA with one molecule of propionyl CoA for the formation of 2-methylbutyrate) in a series of reduction reactions which are analogous to the reversal of mammalian mitochondrial fatty acid ß-oxidation (Komuniecki et al., 1981). The terminal step in the reactions leading to the synthesis of branched chain fatty acids is the reduction of tiglyl CoA's double bond. This reaction is analogous to the ATP producing reduction of fumarate by fumarate reductase. This "branched chain enoyl

reductase" activity is associated with the inner mitochondrial membrane (McKelvey and Fioravanti, 1985). Similar proccesses occuring in molluscs would have great utility.

Hyperosmotic adjustment

A number of organisms including euryhaline polychaetes, bivalves, gastropods, crustaceans (Bricteux-Gregoire et al., 1962; Awapara, 1962; Clark, 1968; Lange, 1972) and non-halophilic bacteria (Measures, 1975) use organic solutes for volume regulation including free intracellular amino acids betaine, TMAO, and the unusual amino acid taurine. The ribbed mussel similarly accumulates free amino acids in response to hyperosmotic stress. In isolated ventricles, alanine, proline, and glycine accumulate in short term exposure, while taurine and quaternary amines change more slowly (Pierce, 1971a). These different species with ability to regulate intracellular free amino acid composition favor different repetoires. For example, while alanine is observed to accumulate most rapidly in the tissue of the ribbed mussel, glycine is most important to the polychaete Arenicola marina (reviewed in Bishop, 1976; Zebe, 1975), and alanine and proline are most important for the intertidal copepod Tigriopus californicus (Burton, 1986).

The accumulating amino acids are probably non-essential and their carbon skeletons can be synthesized from glucose, acetate, and other amino acids (Bishop, 1976). Several fixation sites for amino nitrogen during hyperosmotic adjustment have been suggested (the purine nucleotide cycle: Braunstein, 1957; Bishop and Barnes, 1971; Lowenstein, 1972; serine dehydrase: Gilles,

1969; direct fixation of ammonia via the glutamate dehydrogenase: Reiss et al., 1977; Chaplin et al., 1970).

Increased rate of amino nitrogen fixation in response to hyperosmotic stress by the glutamate dehydrogenase reaction has been suggested for crustaceans (Chaplin et al., 1970; Schoffeniels, 1976). Because glutamate levels do not increase when molluscs are subjected to hyperosmotic stress, increased fixation at glutamate dehydrogenase is unlikely for molluscs (Bishop et al., 1981). However, treatment of ribbed mussel tissues with L-cycloserine or aminooxyacetic acid (two inhibitors of glutamate-linked transaminases: Braunstein, 1957) changes the pattern of free amino acid accumulation. Specifically, aminooxyacetic acid treatment significantly decreases proline and alanine levels while not affecting ß-alanine or taurine levels. Ornithine levels also increase (Bishop and Greenwalt, 1980). These changes in the accumulating amino acid pattern and the slight increase in glutamate level observed in response to aminooxyacetic acid treatment implicate glutamate as an intermediate in the synthesis of many amino acids (Bishop et al., 1981; Baginski and Pierce, 1977). Some of the aminooxyacetic acid sensitive enzymes in ribbed mussel include the mitochondrially localized alanine aminotransferase (Paynter et al., 1984a), and the aspartate aminotransferase which has both a cytosolic and mitochondrial counterpart (Paynter et al., 1984b).

Because aminooxyacetic acid does not affect the magnitude of total amino acid accumulation (Greenwalt and Bishop, 1980) protein breakdown is implicated in contributing free amino acids. The role of protein breakdown is

also suggested by the finding that members of a blue mussel population with a leucine aminotransferase allele with higher enzyme activity accumulate more free amino acids (especially glycine and alanine) than members lacking this allele (Deaton et al., 1984). However, further metabolism of amino acids released from protein catabolism is indicated, because not all amino acids found in protein accumulate (Baginski and Pierce, 1977). Protein derived amino acids might serve as substrate for an L-amino acid oxidase associated with a particulate cell compartment from the ribbed mussel. Consistently, good substrates for this activity include those amino acids which do not accumulate (Burcham et al., 1980). Amino nitrogen released from non-accumulating amino acids by this activity are fixed onto an amino donor for the synthesis of accumulating amino acids by the action of an amino acid synthesizing activity such as glutamate dehydrogenase (Reiss et al., 1977).

While exposure to hyperosmotic medium increases the free amino acid composition, exposure of isolated tissues to hypoosomotic medium causes efflux of free amino acids (Pierce and Greenberg, 1972). Specifically, the concentration of free amino acids remaining within the tissues plus those in the medium is equivalent to the concentration originally present within the tissue. Similar studies on whole organisms show that hypoosmotic stress causes diffusion of free amino acids from the tissues into the hemolymph, where they are distributed to all tissues for slow catabolism (Bartberger and Pierce, 1976). The free amino acid concentration within the ventricle reaches steady state within six hours after transfer to lower salinity (Strange and Crow, 1979b).

Isolated ventricles show only slight changes in water content when subjected to wide ranges of osmotic strength because of the regulation of free amino acid concentration (Pierce and Greenberg, 1972). When M. demissus granosissimus individuals are adapted to either 36‰ and transferred to either 3‰ or 48‰, gains and losses of water content (as assessed by changes in wet weight) of only about 6 % are observed (Pierce, 1971b). The organisms appear to be able to return to original weight when shifted to lower salinity, but original weight is not reestablished in the shift to higher salinity (Pierce, 1971b).

The ionic concentration of the hemolymph from several molluscs also varies with the concentration of the medium. In several oligohaline and euryhaline bivalves (Rangia cuneata, Polymesoda caroliniana, Ostrea palmula, and Polymesoda maritima), hemolymph ionic composition remains hyperosmotic to the medium and fairly constant below 100 mOsm, but above 100 mOsm, the hemolymph ionic composition conforms with the increased ionic strength of the medium (Deaton, 1981). The hemolymph of the ribbed mussel also increases in ionic composition in response to increasing ionic strength of the bathing medium, as well as the mantle fluid and the pericardial fluid (Pierce, 1970), but the hemolymph is hyperosmotic to mantle cavity fluid because of the higher K^+ content in the hemolymph (Strange and Crow, 1979a). The increases are attributed to increased concentrations of Na^+, Ca^{2+}, and Mg^{2+} (Shumway, 1977b). That increasing the ionic concentration of the bathing medium also increases the tissue ionic content has been suggested (Sarkissian and Gomolinski, 1976).

In living intact organisms changes in water content of tissues as assessed by changes in wet weight are under behavioral control, as valve closure retards changes in wet weight when animals are subjected to changing salinity regimes (Shumway, 1977a). In the blue mussel, a linear inverse relationship between hemolymph osmolality and tissue water content is observed only up to 500 mOsm, as this osmolality triggers the valve closure behavioral response (Shumway, 1977a).

The accumulation of free intracellular amino acids in response to increased salinity of the bathing medium may be due at least in part to a depression in metabolism (Baginski and Pierce, 1975) as assessed by decreased glucose derived label in asparate and glutamate and decreased O_2 consumption by isolated ribbed mussel ventricles (Baginski and Pierce, 1978), and decreased CO_2 production from labeled amino acids supplied to crustacean tissue (Gilles, 1979). Altered redox state of the cell in response to depressed metabolism might also regulate the processes resulting in amino acid accumulation by furnishing an intracellular signal (Michaelidis and Storey, 1990).

As changing the ionic composition of the medium causes an alteration in the ionic composition of the hemolymph and possibly the tissues, the changing ions themselves may exert modulatory effects on metabolic enzymes. Most anions are perturbing and their effectiveness in inhibiting enzyme activity is similar for most enzymes examined. Acetate is least effective, followed by Cl^-, with NO_3^- the most effective (Gilles, 1979). Because of the non-perturbing effect of accumulation of organic solutes on enzyme

activity as assessed by lack of effect of K_m and V_{max}, the "compatibility" of these organic osmolytes is indicated (Yancey et al., 1980; Somero and Bowles, 1983). Influx of ions in the initial phases of osmotic adjustments may trigger the alterations in metabolism leading to the synthesis of amino acids and other organic osmolytes for osmotic adjustment, whose presence may then permit a more normal metabolism.

While the signal for commensing the alteration in metabolism has not been defined, the maintenance of the cellular amino acid content is dependent upon membrane mediated events requiring divalent cations. Omission of Ca^{2+} or Mg^{2+} from artificial sea water medium results in the efflux of amino acids and other ninhydrin positive substances, while the non-membrane permeable Ca^{2+} agonist La^{3+} can inhibit this loss (Pierce and Greenberg, 1973).

Evidence for the Importance of Malate Metabolism in Anaerobic and Hyperosmotic Stress

While malate concentration does not change in response to anoxia (Ho and Zubkoff, 1982) and the effect of hyperosmotic stress on malate concentration has not be determined, several lines of evidence indicate that malate is an important intermediate in metabolic adjustments of the ribbed mussel.

Because aspartate serves as a precursor for succinate synthesis during initial adjustment to anaerobic stress, malate is likely to be an intermediate in succinate formation. A pathway for the synthesis of succinate from aspartate

is the cytosolic transamination of aspartate by the cytosolic aspartate aminotransferase (Paynter et al., 1984b), followed by the reduction of the oxaloacetate produced by this reaction by the cMDH. The reduction of oxaloacetate by cMDH, along with the production of opines by the opine dehydrogenases, has been suggested to function in a manner analogous to the mammalian LDH in maintaining the NAD-requiring glyceraldehyde-3-phosphate dehydrogenase reaction of glycolysis (Fields, 1983; Hochachka, 1980).

cMDH has been described in several mollusc species in various states of purity (oyster: Sarkissian and Gomolinski, 1976; blue mussel: Livingstone, 1976; the gastropod Patella cerulea: Lazou et al., 1987). While the cMDH is polymorphic in many mollusc species, no study has attempted to discern the kinetic differences between allozymes.

The appearance of label from $^{14}CO_2$ in succinate implicates malate as a succinate precursor during longer term adjustment to anaerobic stress, as well. Fixation of CO_2 by the cytosolic PEPCK reaction (Paynter et al., 1985a; Harlocker et al., 1991) yields oxaloacetate, which, like the aspartate derived oxaloacetate, may be reduced to malate by the cytosolic malate dehydrogenase.

Malate formed in the cytosol may be transported into the mitochondria. While mitochondria from insect flight muscle and cephalopod mantle muscle possess exchange systems for alpha-glycerol phosphate and dihydroxyacetone (reviewed in Fields, 1983), bivalve mitochondria apparently lack the ability to transport these compounds (Ballantyne and Moon, 1985). Bivalve mitochondria may resemble mammalian mitochondria which

possess a malate/aspartate shuttle because malate serves as an excellent respiratory substrate for these mitochondria (Burcham et al., 1984; Ballantyne and Moon, 1985). Carbon from malate supplied to isolated anaerobic blue mussel mitochondria distributes into many compounds that accumulate under anoxia in intact tissues, especially succinate and propionate (deZwaan et al., 1981; Kluytmans et al., 1975, 1977; Ho and Zubkoff, 1982, 1983).

Some succinate, and therefore probably propionate as well, is formed from malate by dehydration and reduction catalyzed by the fumarase and fumarate reductase activities (deZwaan et al., 1981; Howerda and deZwaan, 1980). The distribution of label from glutamate into succinate during anaerobic incubation indicates that some succinate is formed by some of the forward operating Krebs cycle activities (Collicut and Hochachka, 1977). The anaerobic distribution of label from succinate into aspartate and glutamate implicates the forward operation of other Krebs cycle activities, specifically the mitochondrial malate dehydrogenase, as well as succinate dehydrogenase and fumarase (deZwaan et al., 1981; Wijsman et al.,1977). The forward operating mMDH activity is also implicated in recovery by the succinate dependent increase in O_2 consumption (Malango and Ayello, 1972) and possibly by the conversion of succinate to aspartate (Gäde and Meinardus, 1981). Under ordinary conditions, oxaloacetate synthesis by the mitochondrial malate dehydrogenase of most organisms is limited by the unfavorable equilibrium (Skorkowski et al., 1984). However, this reaction is driven to oxaloacetate formation by oxaloacetate utilization in the citrate synthase reaction of the Krebs cycle (Wijsman et al., 1977). A feature which is usually observed in mitochondria in which mMDH is a major malate metabolizing enzyme is a

sparking of O_2 consumption when pyruvate is added concurrently with malate (Skorkowski et al., 1984). The absence of pyruvate sparking with ribbed mussel mitochondria (Burcham et al., 1984) may suggest that another malate metabolizing enzyme is responsible for the malate dependent O_2 consumption, or that pyruvate is only poorly or not transported across the mitochondrial membrane, or that that there is a source of pyruvate for acetyl CoA production for citrate synthesis other than pyruvate transported from the cytosol. Mitochondrial malate dehydrogenase activity has not been studied previously in molluscs.

While carbon for succinate and propionate synthesis is at the expense of asparate initially and carboxylated PEP later, the source of carbon for alanine synthesis has not been clearly demonstrated. As the isolated mitochondria from bivalves may be incapable of transporting pyruvate (Burcham et al., 1984; Ballantyne and Moon, 1985; Malango and Ayello, 1972), but are capable of using malate as a respiratory fuel (deZwaan et al., 1981), the conversion of four carbon precursor to three carbon product by mitochondrial malic enzyme (Paynter et al., 1985a) may be an important reaction in the synthesis of alanine accumulating during hyperosmotic and anaerobic stress. The possibility that this precursor is aspartate rather than malate is indicated by several observations, including that label from U-^{14}C aspartate appears in alanine in gills from M. demissus subjected to hyperosmotic shift, that the inhibitor of the PEPCK reaction metcaptopicolinic acid slightly decreases the amount of this label appearing in alanine, that the inhibitors of glycolysis iodoacetate and 2-deoxygluocose only inhibit the formation of alanine in response to hyperosmotic stress by 21 and 32%, respectively, and that the inhibitor of

mammalian mitochondrial malate transporter, n-butylmalonate, has no effect on the alanine synthesized in response to hyperosmotic stress (Harlocker et al., 1991; Greenwalt, 1981). The transport of aspartate into the mitochondria might be disadvantageous because the net effect of its transport is oxidation within the mitochondria. However, aspartate transport would make amino nitrogens available for alanine synthesis (Paynter et al., 1984a; 1984b) in addition to possibly providing carbon for its synthesis.

While the ME activities of some alanine producing tissues are quite high, the ribbed mussel has only a very low level of ME activity (Paynter et al., 1985a). This observation plus the observation that U-^{14}C aspartate distributes into alanine (Collicut and Hochachka, 1977), but not 4-^{13}C aspartate (Graham and Ellington, 1985) makes the role of ME in the production of alanine by a simple decarboxylation of a four carbon precursor questionable. An important malic enzyme function in anoxic tolerant parasitic helminths is the production of reduced nucleotides for fumarate reduction (Ascaris suum: Landsperger and Harris, 1976). In several anoxia tolerant organisms in which the malic enzyme is NADP-linked, a co-localized transhydrogenase for the synthesis of fumarate reductase requiring NAD is observed (Hymenolepis sp.: Fioravanti, 1982; M. edulis: Holwerda and deZwaan, 1980). Although the specific activity of the ribbed mussel malic enzyme is low compared to the specific activity reported for parsitic helmithes, the finding that a co-localized transhydrogenase is present in the blue mussel suggests a role for the molluscan malic enzyme in the generation of reduced nucleotides as well.

An alternative function for this malic enzyme might be the synthesis of mitochondrial pyruvate for the PDH reaction (Paynter et al., 1985b). During normal Krebs cycle function, acetyl CoA produced from malate by the ME and PDH reactions may explain the ability of isolated ribbed mussel mitochondria to respire well on malate while possessing only a low activity malic enzyme (Paynter et al., 1985a; Skorkowski et al., 1984). Acetyl CoA formed from malate via the malic enzyme and PDH reactions may also serve as a thiol donor in succinate and propionate formation.

The cellular localization and preliminary kinetic characteristics of malic enzyme from several molluscs have been studied previously (cephalopod: Storey et al., 1975; oyster: Hochachka and Mustafa, 1973; the blue mussel: deZwaan, 1977; deZwaan and vanMarrewijk, 1973; Paynter et al., 1985a; the ribbed mussel: Paynter et al., 1985a).

Because of the importance of malate metabolism in anaerobic and hyperosmotic adjustments made by the ribbed mussel, we herein present the purification and kinetic properties of the cytosolic malate dehydrogenase, the mitochondrial malate dehydrogenase, and the mitochondrial malic enzyme.

CHAPTER 1.

PURIFICATION OF THE MITOCHONDRIAL AND CYTOSOLIC MALATE DEHYDROGENASES FROM RIBBED MUSSEL GILL TISSUE

Abstract: The malate dehydrogenases (MDHs) from the cytosol (cMDH) and the mitochondria (mMDH) of the gill of the ribbed mussel were highly purified. Both are dimers. The native molecular weight of the cMDH is 62,000-65,000, while the molecular weight of the mMDH is 60,000. The cMDH but not the mMDH from ribbed mussel gill cross reacts with antibodies raised to porcine cMDH.

INTRODUCTION

Malate dehydrogenases (MDHs) are typically dimeric enzymes with strong conservation of molecular weight. MDHs from the eubacteriales are among the only forms which have been documented to have larger molecular weight than the 60,000-70,000 observed for the numerous eukaryotic species that have been studied (Kitto and Kaplan, 1966; McReynolds and Kitto, 1970; Englard and Segal, 1969; Murphey et al., 1967). For example, the Bacillus subtilis MDH has a molecular weight of 117,000 (Murphey et al., 1967). In a number of bacterial species, it appears that the 30,000 to 40,000 molecular weight subunit size is conserved, but that many are tetrameric rather than dimeric (Sunduram et al., 1980). Another larger molecular weight MDH has been described from pig heart mitochondria (138,000 MW tetramer) which may dissociate into two proteins of equal molecular weight in the presence of thyroxine (Covelli et al., 1969; Consiglio et al., 1970). This other protein may be a protein other than MDH, thus explaining the presence of the amino acid tryptophan which is normally absent from the mitochondrial MDH (Kitto and Kaplan, 1966; Thorne and Kaplan, 1963; Banaszak and Bradshaw, 1975).

Physical evidence indicates that the subunits are more or less equivalent. Both subunits of an MDH dimer bind nucleotide (Kitto and Kaplan, 1966; Holbrook andWolfe, 1972). Peptide maps produce only half the number of spots predicted on the basis of amino acid composition (Kitto and Kaplan, 1966).

Distinct malate dehydrogenases are associated with the mitochondrial and cytosolic compartments in animal species and a third form is associated with the microbody in plants (Banazsak and Bradshaw, 1975; Ting et al., 1975). The mitochondrial and cytosolic forms are distinct in a number of animal species studied, with the mitochondrial form more closely related to the bacterial MDHs than the cytosolic forms (McAlister-Henn, 1988). That the cMDH of Artemia (a crustacean) is immunologically distinct from the mitochondrial forms of several other organisms has been noted (Hand and Conte, 1982a).

Varying degrees of heterogeneity of MDHs have been observed in mollusc species (Fujio et al., 1983; Ayala, 1973; Buroker, 1983; Koehn and Mitton, 1972). Fujio et al. (1983) report that the number of MDH loci in molluscs ranges from one in Corbicula japanica to five in the oyster. There is a single locus for cMDH in M. demissus with two alleles (Koehn and Mitton, 1972). Because the cytosolic MDH from the ribbed mussel is polymorphic and the forms differ slightly with regard to the affinity for oxaloacetate (Brodey and Bishop, 1991), the partial purification and some of the physical characteristics of the least anodally migrating cytosolic MDH (designated C1 by us and which we have found to be most prevalent in the population from which the animals used in this study are taken) are herein described. The purification and some of the physical properties of the mitochondrial MDH from ribbed mussel are also herein described. Some of the kinetic properties of the cytosolic MDHs from the molluscs Mytilus edulis and Patella caerulea have been studied previously (Livingstone, 1976; Lazou et al., 1987). However, to

our knowledge, there is no information in the literature regarding the properties of the mitochondrial MDH from any mollusc species.

MATERIALS AND METHODS

Animals: Animals were obtained from Northeast Marine Laboratories and maintained as described previously (Greenwalt and Bishop, 1980).

Chemicals and Reagents: Oxaloacetic acid, NADH disodium salt, and most other chemicals were obtained from Sigma (St. Louis), with the following exceptions: monobasic sodium phosphate was obtained from Fisher Scientific (Fair Lawn, N.J.); ammonium sulfate (enzyme grade) was obtained from Schwartz-Mann (Cambridge, Mass.); TEMED, ammonium persulfate, BIS, Affi-gel Blue Gel (100-200 mesh), and nitrocellulose (Trans-Blot) were obtained from Biorad (Richmond, California); agar was obtained from Baltimore Biological Laboratory (Baltimore, MD.).

Enzyme Assay: During enzyme purification, enzyme activity was routinely monitored by addition of enzyme solution to 2.00 ml of a reaction mixture containing 50 mM HEPES (pH 8), 3.03 mM oxaloacetate (20 mg/50 ml), and 258 μM NADH (10 mg/50 ml). Assays were performed at 22° C. The change in absorbance at 340 nm was recorded with a Beckman 3600 recording spectrophotometer.

Enzyme purification: All procedures were performed between 0 and 4° C.

mMDH: Mitochondria from the gills of approximately 40 animals were prepared by the procedure of Burcham et al. (1984). The mitochondria were frozen in 22 ml of 100 mM sodium phosphate (pH 6.8). After thawing, mitochondria were suspended in 100 ml, and sonicated 4 times at 30 seconds

using the highest setting ("8") of a Branson Sonic Power Sonicator with the 1.5 cm diameter probe. Ammonium sulfate powder was added with stirring to 30% saturation. This mixture was stirred slowly for one hour and then centrifuged at 15,000 x g for 20 minutes. Powdered ammonium sulfate was added with stirring to the supernatant to 70% saturation, mixed for one hour, then centrifuged. The pellet was resuspended in 5 ml of 100 mM sodium phoshate (pH 6.8) and dialyzed overnight against this same buffer. The dialyzed sample was applied to a Sephadex G-150 column (36 x 4.5 cm) equilibrated in this same buffer. The fractions containing MDH activity were diluted 1:5 in dd H_2O to reduce the buffer concentration to 20 mM sodium phosphate, pH 6.8. This diluted protein was applied to a DEAE cellulose column (1.5 cm x 24 cm) equilibrated with 20 mM $NaPO_4$, pH 7.0. The column was washed with 85 ml of this same buffer. The malate dehydrogenase activity was eluted with a 0-200 mM KCl gradient (total volume =300 ml) in the same buffer. Active fractions were pooled and dialyzed against 20 mM $NaPO_4$, pH 7.0, and applied to an Affi-Gel Blue column (1.0 cm x 6 cm) equilibrated in the same buffer. The column was washed with 30 ml of the same buffer and MDH activity was eluted with a 0.5 M KCl solution in this same buffer. Active fractions were pooled, dialyzed against storage buffer (20 mM Tris, 20% glycerol, 0.5 mM DTT) and frozen at -20°C until ready for assay.

C1 Isozyme: The C1 (least anodally migrating at pH 8.8) isozyme was partially purified for the kinetic studies reported in the following chapter. Gill tissue from approximately 20 animals was homogenized and mitochondria removed as described in Burcham et al. (1984). The

supernatant from the 9,000xg centrifugation ("cytosol") was made 30 % saturated with powdered ammonium sulfate and stirred slowly for one hour. This mixture was centrifuged for 20 minutes at 15,000xg. To the supernatant, additional powdered ammonium sulfate was added to 70 % saturation. The precipitated material was collected by centrifugation at 9,000 x g for 20 minutes. As both the 30 % saturated and 70 % saturated pellets had measurable MDH activity, these pellets were pooled and dissolved in 5 mM sodium phosphate buffer (pH 6.4), dialyzed against two changes (2 liters each) H_2O and once against 2 liters 5 mM sodium phosphate buffer (pH 6.4). This material was applied to a well equilibrated DEAE cellulose (Sigma, medium mesh) column (1.5 x 24 cm). The column was washed with 100 ml 5 mM sodium phosphate buffer (pH 6.4). The MDH activity was not observed to stick to this column, as there was substantial MDH activity in the load and wash fractions and no activity could be recovered with the application of a 400 ml 0-500 mM NaCl gradient in this same buffer. The fractions in the load and wash steps which possessed MDH activity were dialyzed against 5 mM sodium phosphate (pH 6.5) and applied to a second equilibrated DEAE cellulose column. The protein was applied to a second DEAE column, and the column was again washed with 100 ml sodium phoshate buffer (pH 6.4). A salt gradient (0-500 mM NaCl in 5 mM sodium phosphate) was applied to the column. The electrophoretic identity of the two peaks was determined by native gel electrophoresis. The partially purified cytosolic preparation was dialyzed against 20 mM sodium phosphate and was applied to an Affi-gel Blue column (2.5 x 10 cm). The column was washed with 125 ml of this same buffer, then the MDH activity was eluted with the application of a 0 to 2 M

NaCl gradient in this same buffer with a total volume of 300 ml. This preparation was concentrated on an Amicon PM 10 membrane (35 ppi, N_2). A sample was taken for analysis during the concentration step. When the sample volume had been reduced to 5 ml, the sample was loaded onto a Sepharose 6B CL column (1.5 x 96 cm) equilibrated in 20 mM sodium phosphate (pH 7.0). Active fractions were mixed 1:1 with a buffer consisting of 20 mM Tris, 20 % glycerol, and 0.5 mM DTT, then applied to a small Reactive Red column (0.5 cm by 4.5 cm) equilibrated in this same buffer. The column was then washed with 15 ml of this same buffer and MDH activity was recovered by application of 2 M sodium chloride in this same buffer. This preparation was dialyzed against a buffer containing 20 mM Tris (pH 7), 20% glycerol, and 0.5 mM DTT and stored in small aliquots at -20°C.

Protein Concentration: Protein concentrations were routinely performed using Miller's (1959) modification of Lowry's method. For purified preparations in glycerol containing buffers, Spector's modification (1978) of Bradford's method was used.

Gel Electrophoresis: Polyacrylamide gel electrophoresis in the presence of SDS was routinely performed on 12.5 % polyacrylamide gels according to the method of Laemmli (1970). Protein bands were stained with Coomassie Brilliant Blue R250. Identification of the post DEAE cellulose peaks as the C1 and mitochondrial enzymes was made by electrophoresing the protein on a 7 % polyacrylamide gel using Tris (pH 8.8) as the gel buffer and Tris-glycine as the running buffer. MDH activity was identified by the method of Harris and Hopkinson (1976)

Native Molecular Weight Determinations: A Sephadex G-150 column (2 x 42 cm) equilibrated in 100 mM sodium phosphate (pH 6.8) was calibrated and used to determine the native molecular weight of the post DEAE cellulose C1 MDH. To determine the native molecular weight of the mMDH, an aliquot of the concentrated ammonium sulfate precipitated mitochondrial sonicate was applied to a calibrated Sephadex G-150 column (4.5 x 36 cm) equilibrated in 100 mM $NaPO_4$ (pH 6.8).

Preparation of Anti-Porcine cMDH Antibodies: Porcine cMDH was obtained from Sigma. An aliquot (50 µl containing 0.65 mg) was dialyzed against H_2O and mixed with 1.4 ml of Freund's complete adjuvant. Following the removal of approximately 5 ml of whole blood from the ear vein for preimmune serum, this solution was injected subcutaneously between the shaven shoulder blades of a young female white rabbit. Thirty-three days later, the procedure was repeated. After an additional 2 weeks, a blood sample was taken from the ear vein. The collected blood was incubated at room temperature for 3 hours in a plastic centrifuge tube to allow clotting to occur. The clotted material was removed by centrifugation in a table top clinical centrifuge for 30 minutes. The presence of antibodies in this preparation was tested by reactivity with the porcine MDH by Ochterlony double diffusion test performed in borate saline buffer with 1% agar (Campbell et al., 1970). The following day, blood was collected by cardiac puncture, and clots were removed as described above. This preparation was stored at -20° C.

Western Blotting and Detection: Cross reactivity of antibodies raised to porcine heart cMDH with ribbed mussel cytosolic proteins separated on an SDS gel and transferred to nitrocellullose (western blotting) would demonstrate structural similarities between these cMDHs and would also permit determination of the subunit size of the ribbed mussel cMDH. All western blotting procedures were performed at 22°C. Western blotting was performed on proteins separated on 12.5 % SDS polyacrylamide gels (Laemmli, 1970). Prior to blotting, gels were prefixed by soaking for 30 minutes in transfer buffer (25 mM Tris base, 192 mM glycine, and 20 % (v/v) methanol). Blotting onto nitrocellulose strips was performed at 100 mA for five hours using a Biorad Transblot apparatus. After blotting, the nitrocellulose was blocked with 5 % w/v powdered dry milk in Tris buffered saline (10 mM Tris, pH 7.4, 0.9% NaCl). After 2 hours, the blocking solution was supplemented with the antiserum (1:500) and allowed to react for another 6 hours. This solution was removed and the strips were washed with several changes of Tris buffered saline. For detection of the bound rabbit anti-porcine heart cMDH IgG to the western blot, a secondary goat anti-rabbit IgG labeled with horse raddish peroxidase was used in conjuction with 4-chloronapthol and hydrogen peroxide to produce staining on the western blot over the region of the ribbed mussel MDH. Another 5 % milk solution supplemented with goat anti-rabbit IgG-horse radish peroxidase conjugated antibody (1:2000) was allowed to react for 6 hours with the nitrocellulose strips. This solution was then removed and the strips were again rinsed with several changes of Tris buffered saline. The strips were then added to fresh peroxidase substrate solution (50 mg 4-chloronapthol and 40 µl hydrogen

peroxide in 17 ml ethanol and 83 ml of water). This reaction was allowed to proceed for one hour. Black reaction product deposited on the nitrocellulose indicated the area of cross-reactivity.

Test for Antibody Specificity: To test for specificity of the anti-porcine cytosolic MDH antibody, the antibody preparation was reacted with nitrocellulose strips onto which 125 μg of total cytosolic protein and 125 μg of total mitochondrial protein (from cytosol and sonicated mitochondria prepared as described above) had been transferred. Replicate strips of blots of electrophoresed cytosol and mitochondria were probed with preimmune serum.

Inactivation of cMDH in a cytosol preparation with affinity purified antibodies was performed with a procedure modified after Olmsted (1981). To cytosol (the 9,000 x g supernatant from the mitochondrial preparation) sufficient 5x sample buffer was added to adjust the final protein concentration in the sample buffer to 2.5 μg/μl. Two hundred microliters of this protein solution (=500 μg) were applied to each of eight lanes of a 12.5% polyacrylamide SDS gel. Prestained molecular weight standards were applied to the outer lanes. Following the electrophoretic separation, the gel was fixed for blotting in 25 mM Tris base, 192 mM glycine, and 20% v/v methanol. A strip (5 cm in width) in the region of the 35,000 molecular weight marker was blotted with the transblot apparatus as described above. The entire blot was incubated with the rabbit anti-porcine cytosolic MDH (1:500 antibody dilution) in 5% w/v powdered dry milk in Tris buffered saline. A portion of the blot corresponding to the standard lane and one of the cytosol lanes was removed

and reacted with goat-anti rabbit IgG HRP conjugate (1:2000), then stained for HRP activity with 4-chloronaphthol and hydrogen peroxide. The unstained remainder of the blot was washed in Tris buffered saline to remove unbound antibody and other serum components. This strip was compared with the stained portion of the blot; the area of the blot corresponding to the stained region was excised with an exacto knife and minced slightly. The minced nitrocellulose was placed in a three milliliter syringe with 3 ml of 0.2 M glycine-HCl (pH 2.8) at room temperature. The syringe was capped and the slurry was mixed for 10 minutes. The liquid was then drawn into the syringe and squirted out several times for additional mixing. The liquid was then transferred to a dialysis tube and the preparation was dialyzed overnight at 4°C against 400 ml Tris buffered saline.

These affinity purified antibodies were used for the inactivation studies with the mussel cMDH (see Results). A 1:2 dilution series was made from the dialyzed pellet using Tris buffered saline. An aliquot of each (25 µl) was placed in a test tube along with 50 µl TBS and 50 µl of the antibody preparation. The mixtures were incubated for 10 minutes at 23°C then rapidly placed on ice and assayed by adding 50 µl to one ml assay mixture composed of 50 mM HEPES (pH 8.0), 3 mM oxaloacetate, and 266 µM NADH. MDH activity was measured spectrophotometrically as described above. Inactivation was tested by comparing the slopes of antibody-incubated diluted enzyme preparations with diluted enzyme preparations to which a volume equivalent to the volume of antibody was added (composed of 25 µl of the enzyme plus 100 µl of TBS). To test for MDH activity of the diluted antibody, a dilution of the antibody preparation was made (50 µl antibody plus 75 µl

TBS) and assayed for MDH activity. To test the effect of antibody dilution on the inactivation of MDH, a small range of 1:2 dilutions of the antibody preparation was made. Aliquots (25 µl) of the 1:256 dilution of the enzyme were transferred to test tubes along with 25 µl of the antibody dilution series plus 50 µl of the TBS. Controls consisting of antibody of the proper dilution plus 50 µl of TBS were also assayed. All of these were incubated and assayed as described above.

RESULTS

The mMDH activity from the ribbed mussel gill tissue elutes as a single peak from the Sephadex G-150 column (Fig. 1), from DEAE cellulose (Fig. 2), and from the Affi-gel Blue column (Fig. 3). The result of this three column purification was a partially purified preparation with high specific activity (Table 1) which produced a major band and a larger molecular weight minor band on an SDS gel (Fig. 8). A similar procedure was used to partially purify the cMDH from ribbed mussel gill tissue. The initial DEAE cellulose steps produced a good purification and also permited the separation of the cMDH from contaminating mMDH released from mitochondria broken during the homogenization step (Fig. 4). The cMDH activity elutes as symmetrical peaks from Affi-gel Blue (Fig. 5) and from Sepharose 6B CL (Fig. 6). The Reactive Red step did not produce any improvement in specific activity (Table 2).

The cMDH and mMDH from ribbed mussel have similar molecular weights as determined by gel filtration on calibrated Sephadex G-150 columns (Figs. 7 and 8). The molecular weight of the mitochondrial form was determined to be 60,000 while the native molecular weight of the cMDH is 65,000 using Sephadex G-150 columns.

The preimmune serum was unable to react with any protein present in the cytosol or mitochondria, as revealed by western blotting. Antibodies raised to the native porcine cMDH cross reacted with the porcine cMDH, as revealed by Ochterlony double diffusion plate (data not shown). Western blotting revealed that antibodies to porcine cMDH reacted with a 32,000 MW

Figure 1. Elution Profile of Mitochondrial Malate Dehydrogenase from the Ribbed Mussel Gill Tissue on Sephadex G-150. Ammonium sulfate precipitated mitochondrial sonicate was applied to a well equilibrated (100 mM sodium phosphate, pH 6.8) Sephadex G-150 column (4.5 x 36 cm). Fractions were analyzed for protein content (A_{280}; open squares) and MDH activity as NADH-dependent oxaloacetate reducing activity (closed circles). For conditions, see Materials and Methods.

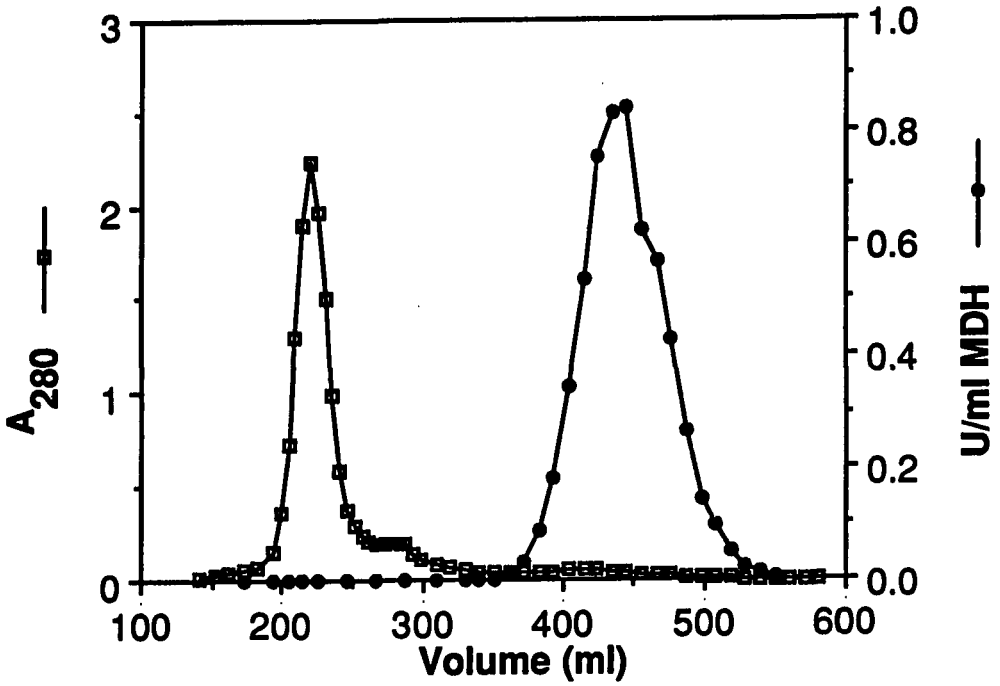

3
2
1
0
A_{280}
1.0
0.8
0.6
0.4
0.2
0.0
U/ml MDH
100
200
300
400
500
600
Volume (ml)

Figure 2. DEAE Cellulose Chromatography of mMDH from Ribbed Mussel Gill. Mitochondrial malate dehydrogenase activity from the Sephadex column was diluted 1:1 with H_2O and applied to a well-equilibrated DEAE cellulose column (1.5 x 24 cm)(See Materials and Methods). Malate dehydrogenase was eluted with a linear salt gradient (0-200 mM KCl; closed circles). The arrow denotes start of the gradient. The active fractions were identified by standard assay (open squares).

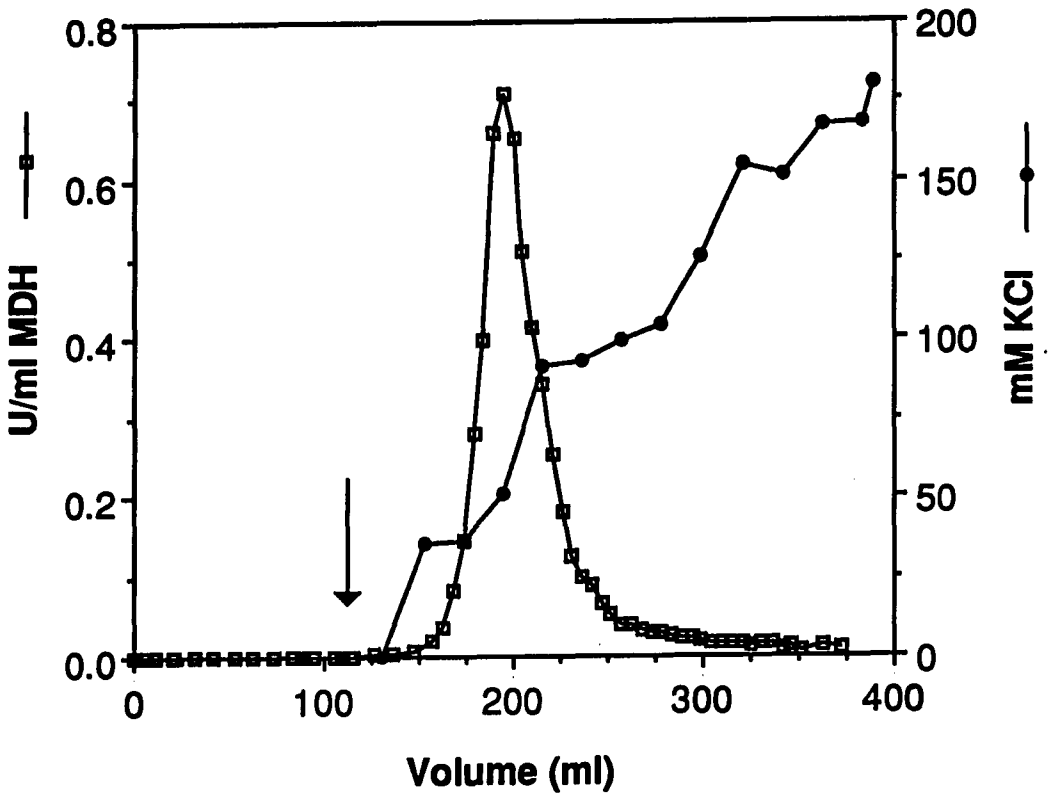

0.8
0.6
0.4
0.2
0.0
U/ml MDH
200
150
100
50
0
mM KCl
0
100
200
300
400
Volume (ml)

Figure 3. Affi-gel Blue Chromatography of the Ribbed Mussel mMDH. The dialyzed solution was applied to an equilibrated (20 mM sodium phosphate, pH 7.0) Affi-gel blue column (1 x 6 cm). The column was washed with 30 ml buffer and mitochondrial malate dehydrogenase was specifically eluted with a solution supplemented with 0.5 M KCl (denoted by arrow).

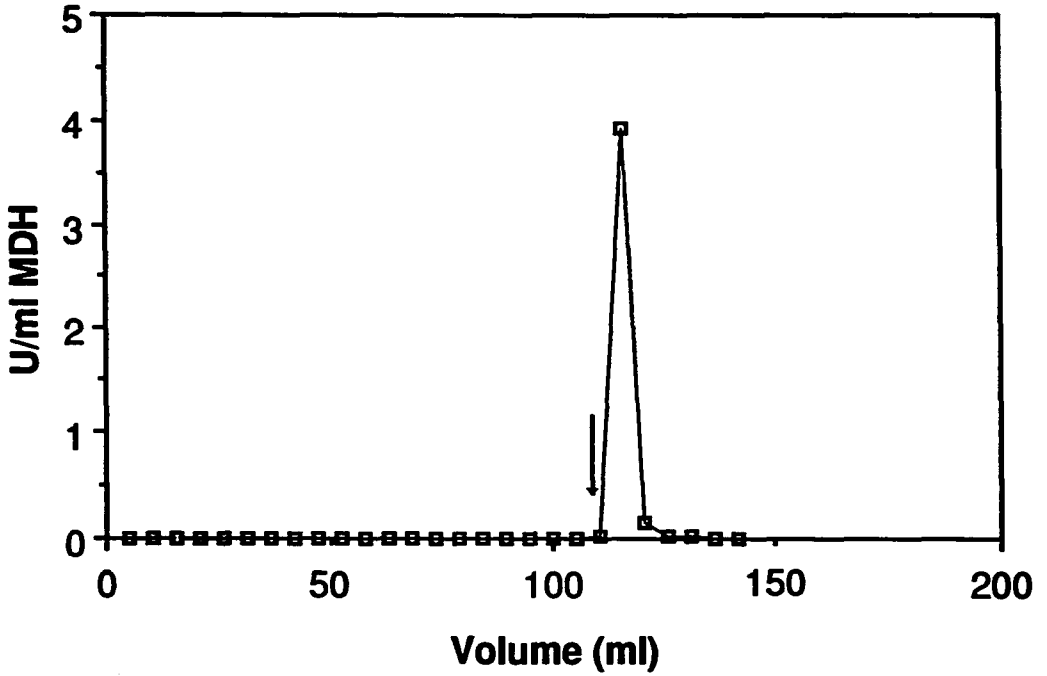
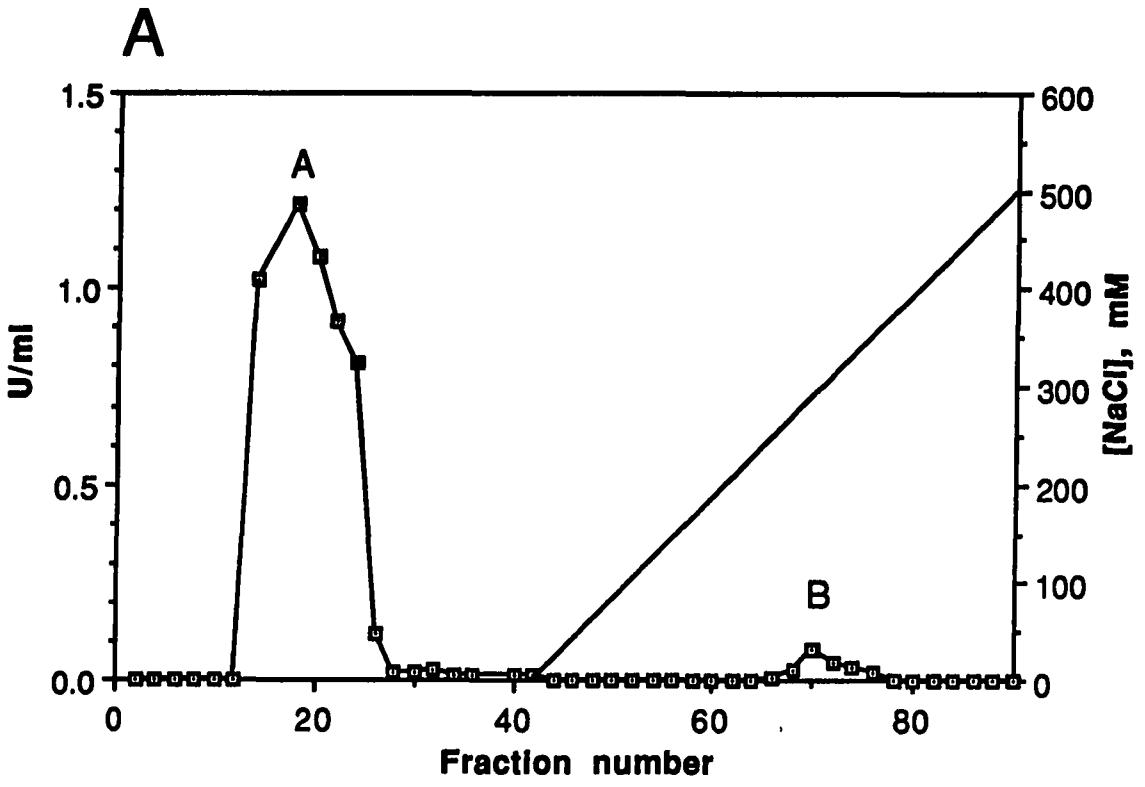

5
4
3
2
1
0
U/ml MDH
0
50
100
150
200
Volume (ml)

Figure 4. Separation of the mMDH from the cMDH with DEAE Cellulose Chromatography.

A. Elution profile of MDH activity from DEAE Cellulose. Peaks A and B are identified below.

B. Native starch gel pattern of MDH's eluted from the DEAE cellulose column. Lane 1: Ammonium sulfate precipitated cytosol. Lane 2: Ammonium sulfate precipitated mitochondrial proteins. Lane 3: Peak B from the DEAE cellulose column. Lane 4: Fraction 14 from the DEAE cellulose column. Lane 5: Fraction 20 from the DEAE cellulose column. Lane 6: Fraction 24 from the DEAE cellulose column.


A
1.5
1.0
0.5
0.0
U/ml
A
B
600
500
400
300
200
100
0
[NaCl], mM
0
20
40
60
80
Fraction number

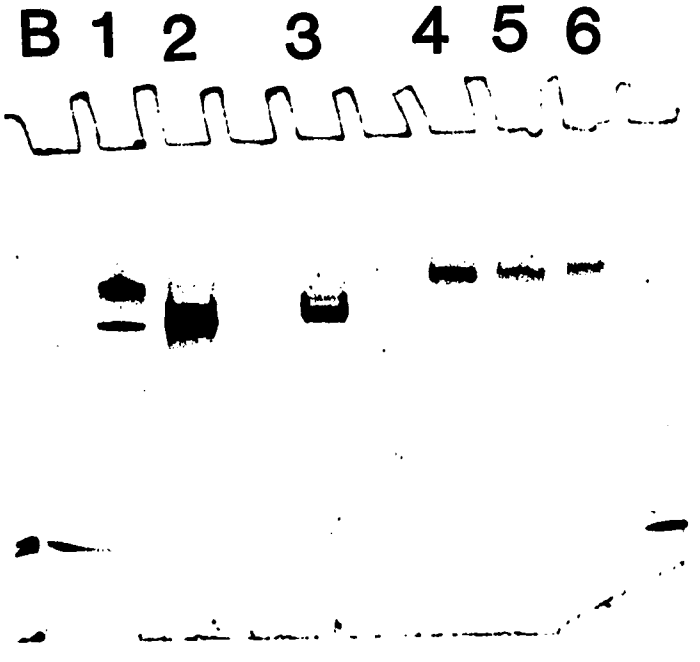

B 1 2 3 4 5 6

Figure 5. Elution of cMDH from Affi-gel Blue. cMDH activity was loaded onto the column, the column was washed with buffer, and the cMDH activity was eluted with the application of a linear NaCl gradient. See Materials and Methods for conditions.

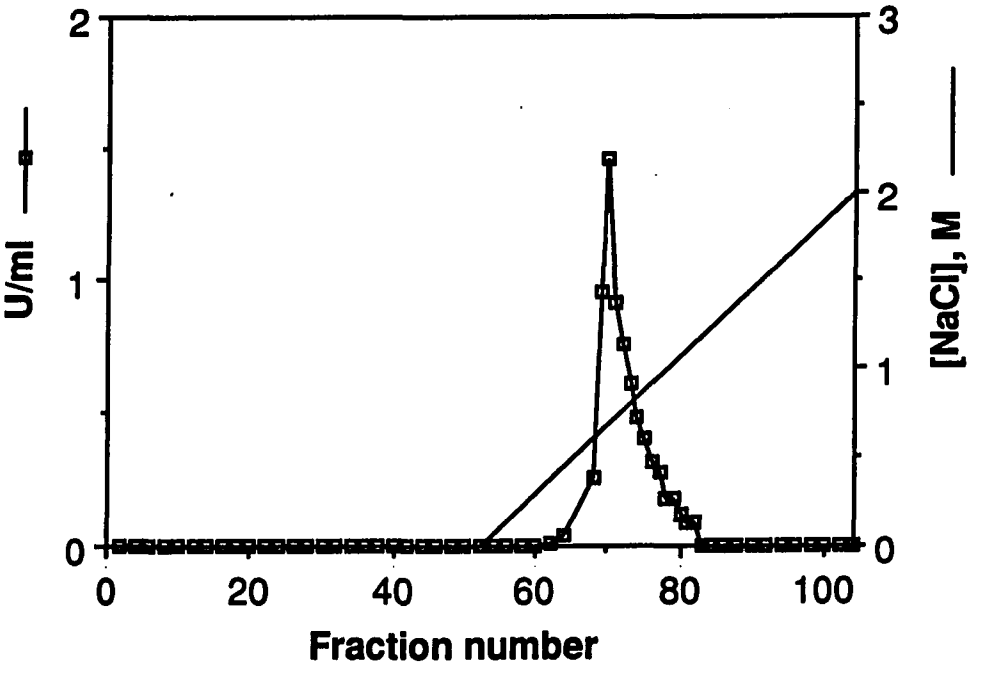

2
1
0
U/ml
3
2
1
0
[NaCl], M
0
20
40
60
80
100
Fraction number

Figure 6. Elution of cMDH from Separose 6B CL. (See Materials and Methods for conditions.)

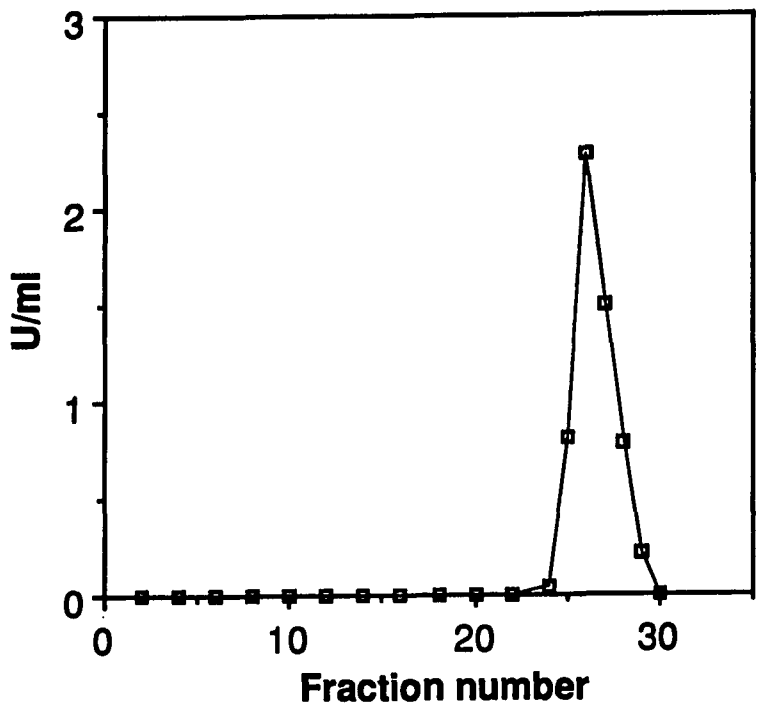

3
2
1
0
U/ml
0
10
20
30
Fraction number

Figure 7. Native Molecular Weight of the C1 Isozyme Determined by Gel Filtration on Sephadex G-150. (See Materials and Methods for conditions).

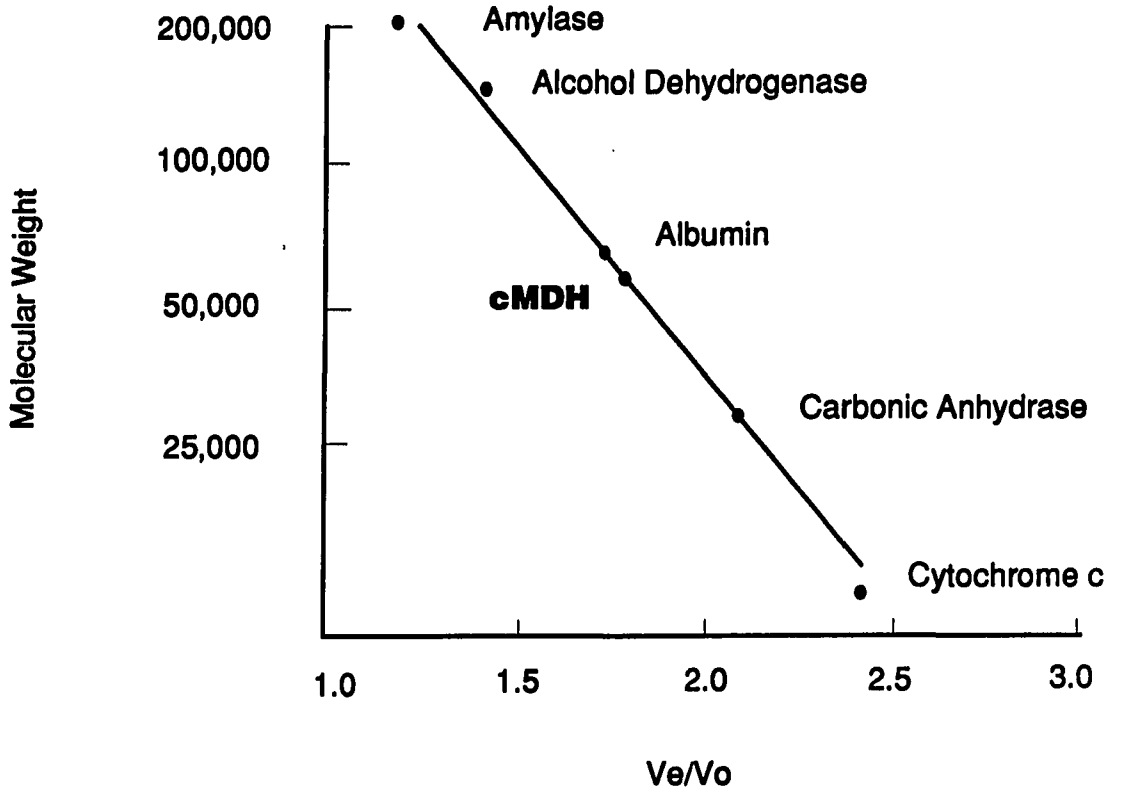

200,000
100,000
50,000
25,000
Molecular Weight
Amylase
Alcohol Dehydrogenase
Albumin
cMDH
Carbonic Anhydrase
Cytochrome c
1.0
1.5
2.0
2.5
3.0
Ve/Vo

Figure 8. Native Molecular Weight Determination of the Mitochondrial MDH by Gel Filtration on Sephadex G-150. (See Materials and Methods for conditions). Inset: SDS polyacrylamide gel showing the result of the three column purification.

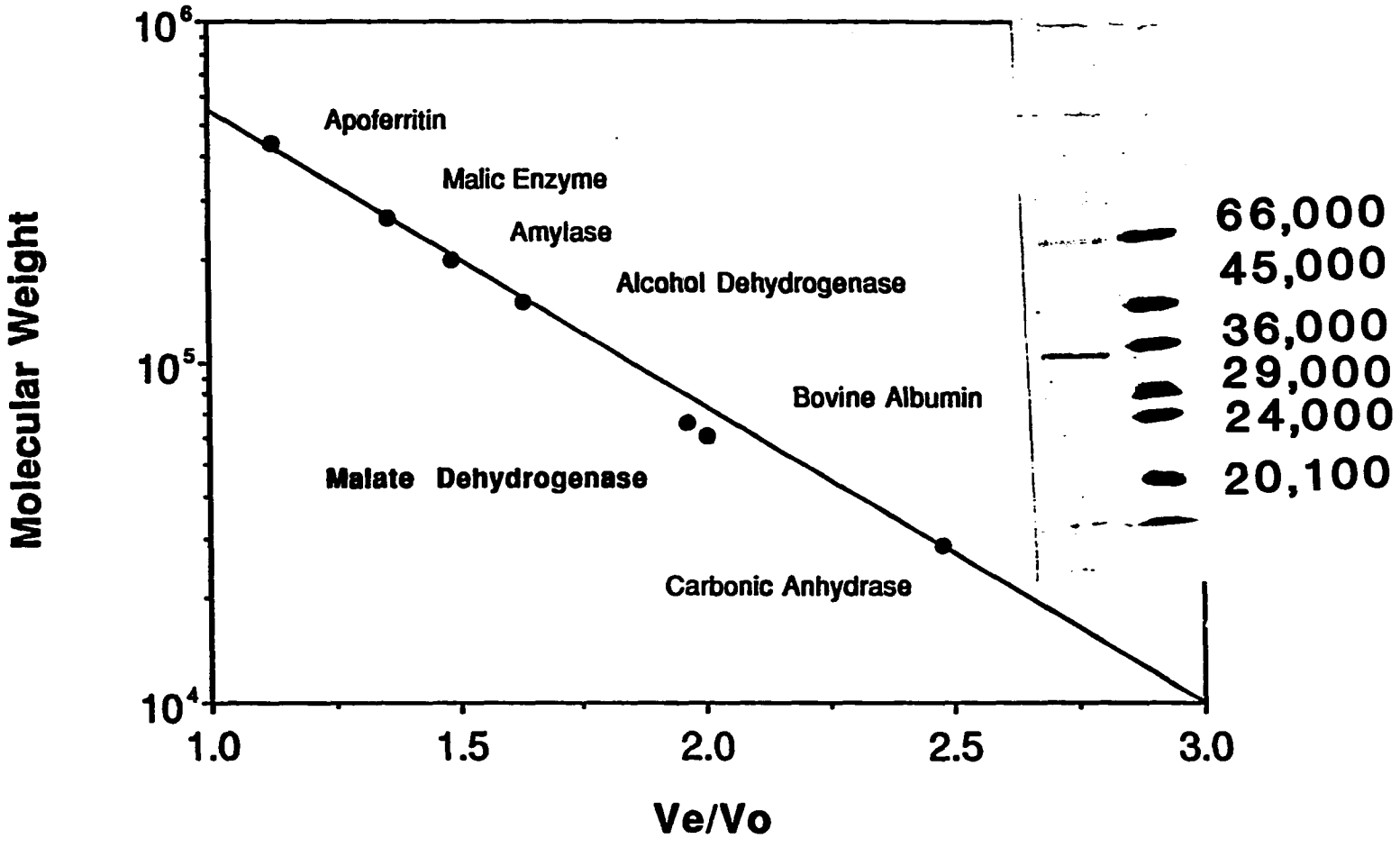

Molecular Weight
10^6
10^5
10^4
Apoferritin
Malic Enzyme
Amylase
Alcohol Dehydrogenase
Bovine Albumin
Malate Dehydrogenase
Carbonic Anhydrase
1.0
1.5
2.0
2.5
3.0
Ve/Vo
66,000
45,000
36,000
29,000
24,000
20,100

Figure 9. Western Blot of Cytosolic Fraction Probed with Rabbit Anti-Porcine MDH. (See Materials and Methods for conditions). Molecular Weight Standards shown at left are expressed as M_r values x 10^{-3}.

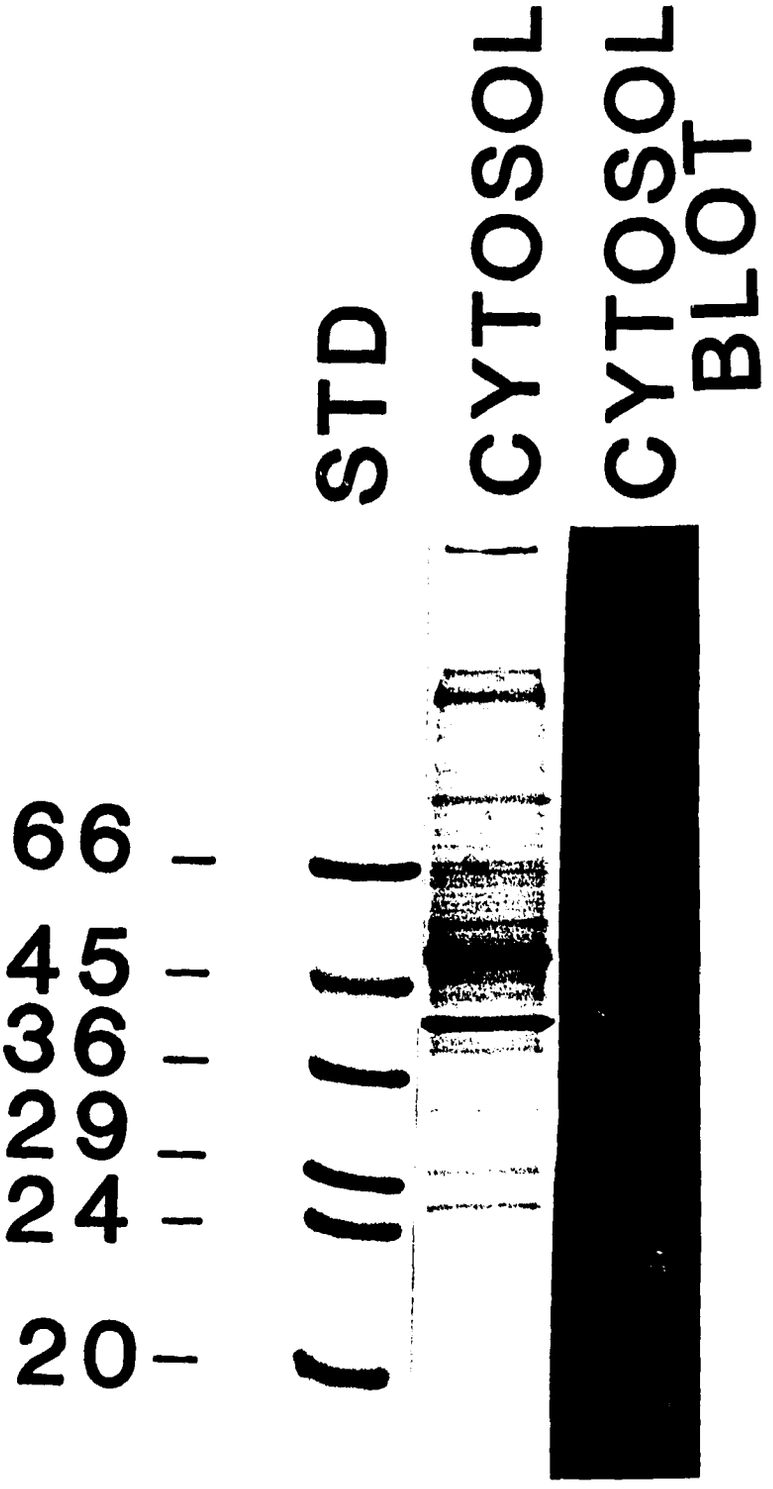
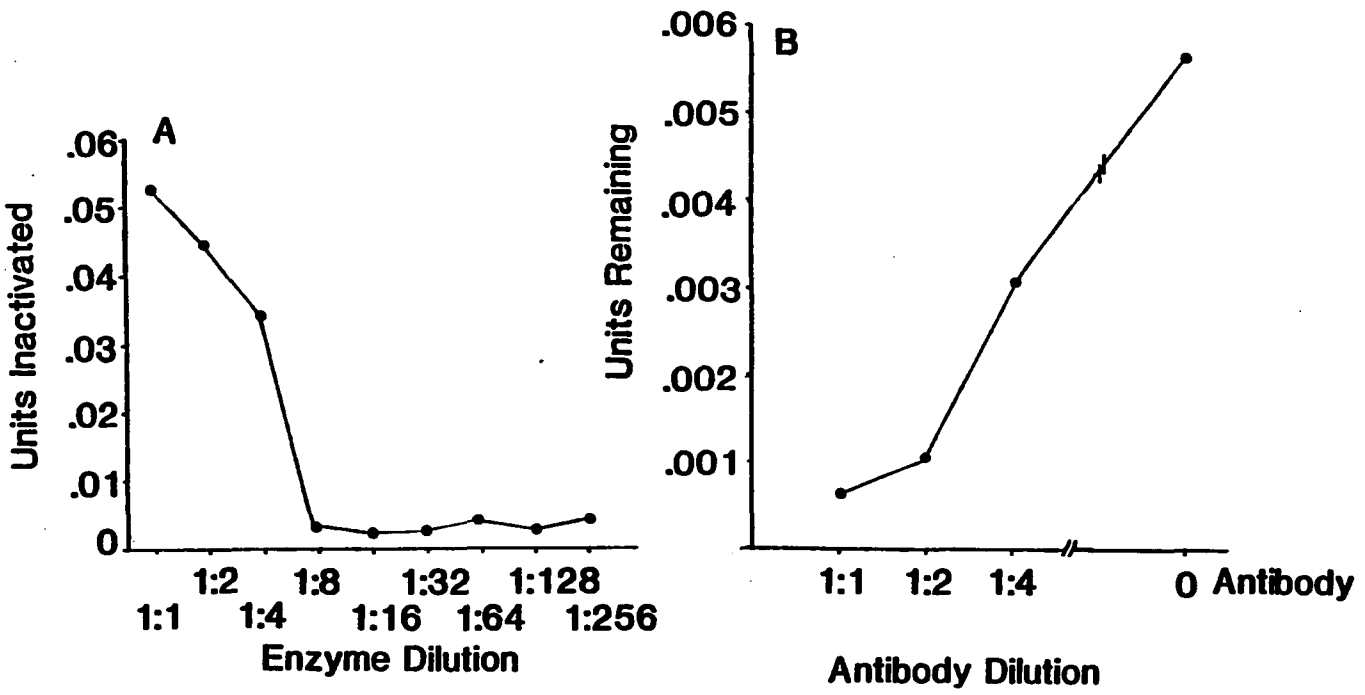

STD
CYTOSOL
CYTOSOL
BLOT
66
45
36
29
24
20

Figure 10. Anti-porcine cMDH Inactivates the Ribbed Mussel cMDH. Affinity purified anti-porcine cMDH antibodies were tested for their ability to inactivate the ribbed mussel gill cMDH from a crude cytosolic preparation. Conditions are described in Materials and Methods.

Part A: Effect of decreasing enzyme concentration.

Part B: Effect of decreasing antibody concentration.


A
Units Inactivated
.06
.05
.04
.03
.02
.01
0
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
Enzyme Dilution
B
Units Remaining
.006
.005
.004
.003
.002
.001
1:1
1:2
1:4
0 Antibody
Antibody Dilution

protein found only in the cytosolic fraction of the gill tissue and not in the mitochondrial portion (Fig. 9). Rabbit anti-porine heart cytosolic antibodies cross-reacting with the 32,000 molecular weight band from the ribbed mussel were removed from the nitrocellulose and collected as described in the Materials and Methods section. These affinity-purified antibodies inactivated MDH activity from ammonium sulfate precipitate of the cytosolic fraction (Fig. 10), indicating that this 32,000 molecular weight band is the ribbed mussel gill cMDH. The affinity purification procedure was necessary to remove the high amount of endogenous rabbit serum MDH activity found in the serum preparation used as the antibody source

Table 1. Purification of mitochondrial malate dehydrogenase.

step	volume (ml)	U/ml[a]	total activity	[protein] mg/ml	U/mg	yield	purification
crude mitochondria	22	3.1	69	5.4 mg/ml[b]	0.58	100%	1x
ammonium sulfate	10	5.7	57	3.6 mg/ml[b]	1.60	84	3
Sephadex	74	0.29	22	0.033 mg/ml[b]	9	32	15
DEAE	52	0.22	11	0.002 mg/ml[b]	109	17	189
Affi-gel Blue	11	0.99	10	0.006 mg/ml[c]	157	15	271

[a]One unit (U) is defined as that amount capable of producing the formation of 1 µmole of product per minute.

[b]Protein was determined by the method of Lowry.

[c]Protein was determined by the method of Bradford.

Table 2. Purification of cytosolic malate dehydrogenase.

step	volume ml	U/ml[a]	total activity	[protein] mg/ml	U/mg	yield	purification
crude cytosol	142	1.021	144.98	3.51 mg/ml[b]	0.291	100%	1x
ammonium sulfate	60	2.432	145.92	2.14 mg/ml[b]	1.136	100%	3.90
DEAE	130	0.590	76.70	0.113 mg/ml[b]	5.221	52.90%	17.94
Affi-gel	30	1.829	54.87	0.0275 mg/ml[b]	66.51	37.85	228.56
Sepharose	38	1.087	41.31	.0115 mg/ml[c]	90.58	28.49	311.27
Reactive Red	13	1.372	17.84	.0166 mg/ml[c]	80.71	12.31	277.32

[a]One unit (U) is defined as that amount capable of producing the formation of 1 µmole of product per minute.

[b]Protein was determined by the method of Lowry.

[c]Protein was determined by the method of Bradford.

DISCUSSION

The native molecular weights for the cMDH and mMDH are approximately 60,000-65,000. The purified mitochondrial enzyme produces a major band on SDS polyacrylamide gels with molecular weight of about 30,000, while the cytosolic enzyme has a molecular weight on western blots of SDS gels of 32,000 (Figs. 7 and 8). As with other malate dehydrogenases, the gill cMDH and mMDH are dimeric (Figs. 7 and 8). These values are slightly smaller than the molecular weights observed for other invertebrate malate dehydrogenases, specifically, 75,000 for the cytosolic MDH of the foot of Patella caerula (Lazou et al., 1987), and 72,000 for the cytoslic MDH of Artemia nauliar larvae (Hand et al., 1981).

That the cytosolic and mitochondrial forms of the malate dehydrogenases from the ribbed mussel are immunologically distinct (Fig. 9) is consistent with the observations made by several others on the immunoreactivity of forms from the cell compartments of other organisms. For example, the monospecific antiserum raised to the cMDH of Artemia larvae cannot cross-react with the mMDHs from unspecified tissues of pig, beef, and pigeon (Hand and Conte, 1982a). Similarly, Kaplan and Kitto (1966) report that the antiserum raised to purified chicken cMDH did not cross-react with the mMDH, whereas anti-mMDH antiserum can cross-react with cytosolic forms (Hägele et al., 1978; Grimm and Doherty, 1961). Antibodies raised to the cMDH from Artemia nauplii have been used to show that MDH levels decrease as the organism metamorphoses from the emergent stages to the naupliar stage when the organism is held at constant salinity and that the

amount of enzyme does not fall when the organism is exposed to elevated salinity (Hand and Conte, 1982b).

Results presented here demonstrate that some of the physical properties of the cMDH and mMDH from the ribbed mussel are similar to those observed for many diverse eukaryotic species. Results presented here also confirm that the separation of the cytosolic and mitochondrial MDHs (McAlister-Henn, 1988) holds true for the molluscs, as the cytosolic and mitochondrial forms are immunologically distinct and the cytosolic form is cross reactive toward anti-cytosolic porcine MDH antibodies.

CHAPTER 2.

KINETIC PROPERTIES OF THE CYTOSOLIC AND THE MITOCHONDRIAL MALATE DEHYDROGENASES FROM THE RIBBED MUSSEL GILL

Abstract: The kinetic properties of highly purified preparations of malate dehydrogenase from the cytosol (the least anodally migrating form, cMDH) and mitochondria (mMDH) of the gill tissue of the ribbed mussel were studied under several conditions. At pH 8, the apparent K_m's for OAA were 10 μM and 8 μM for the cMDH and mMDH, respectively. At pH 8, the apparent K_m's for NADH were 6 μM and 19 μM for the cMDH and mMDH, respectively. Because of the unfavorable equilibria, malate and NAD kinetics were studied at pH 9.2. At this pH, the malate apparent K_m's were 0.46 mM and 1.06 mM for the cMDH and mMDH. The apparent K_m's for NAD were 38 μM and 151 μM for the cMDH and mMDH. At pH 9.2, the apparent K_m's for OAA were increased to 105 μM and 47 μM for the cMDH and mMDH. At pH 9.2, the apparent K_m's for NADH were increased to 17 μM and 35 μM for the cMDH and mMDH. Inclusion of 10 mM $MgCl_2$ in the pH 9.2 assay mixtures raised the apparent K_m's for OAA for the cMDH and mMDH to 571 μM and 132 μM. In the presence of 10 mM $MgCl_2$, the apparent K_m's for NADH were 30 μM and 35 μM for the cMDH and the mMDH. The inclusion of $MgCl_2$ raised the apparent K_m's for malate to 0.831 mM and 1.35 mM for the cMDH and mMDH. The inclusion of $MgCl_2$ also increased the apparent K_m for NAD to 45 μM and 189 μM. The mMDH was more sensitive to inhibition by sodium chloride, potasium chloride, and lithium chloride than the cMDH when assayed in the OAA utilizing direction. While sodium acetate was much less inhibitory to the mMDH than the other salts tested, it inhibited the cMDH about equally as the chloride salts tested. When assayed in the OAA utilizing direction, both cMDH and mMDH were inhibited by the dicarboxylic acids malate, succinate, and aspartate in decreasing order of effectiveness.

Alanine was not inhibitory to the mMDH, but was found to inhibit the cMDH. When assayed in the OAA utilizing direction, hydroxymalonate inhibited the mMDH competitively with an apparent K_{islope} = 0.256 mM and inhibited the cMDH non-competitvely with an apparent K_{islope} = 1.172 mM. ATP inhibited the cMDH competitively (K_{islope} = 0.77 mM), and the mMDH in a mixed non-competitive manner (K_{islope} = 5.38 mM). The pH optimum of the mMDH for the OAA utilizing direction was slightly higher than the pH optimum of the cMDH (8.5 vs. 7.5), while the two forms had similar pH optima for malate utilization.

INTRODUCTION

Marine molluscs accumulate high levels of free amino acids in response to hyperosmotic stress (Pierce, 1982). In the ribbed mussel, alanine is the most important of these osmolytes during short term osmotic adjustment, as its levels are observed to increase the most dramatically in response to this stress (Baginski and Pierce, 1978). Alanine is also observed to accumulate in this organism in response to anaerobic stress (Ho and Zubkoff, 1982, 1983), a condition for which this organism is apparently well-suited, as it is found at the highest point in the intertidal zone of any of the bivalves (Lent, 1968). Many invertebrate species lack or have very low levels of active L-lactate dehydrogenase, an activity which classically has been presumed necessary in vertebrates for maintaining cellular redox balance (see Fields, 1983). In response to anaerobic stress, the ribbed mussel, like many other invertebrates with anaerobic capacity such as parasitic helminths, synthesizes succinate and propionate as the major metabolic endproducts, plus some other volatile fatty acids and some branched chain fatty acids (Ho and Zubkoff, 1982, 1983; de Zwaan, 1977; and Saz, 1981).

An immediate response to anaerobic exposure has been suggested to be the production of malate by the cytosolic malate dehydrogenase (Hochachka et al., 1973). Malate has the capacity of traversing the mitochondrial membrane and serving as an anerobic fuel source for anaerobic mitochondria (Burcham et al., 1984; Ballantyne and Moon, 1985; deZwaan et al., 1981). Once inside the mitochondrion, malate has several possible fates and understanding the enzyme activities which catalyze the malate dependent reactions would allow

a better understanding of the contribution that malate makes to the endproducts which accumulate in response to hyperosmotic stress and anaerobiosis.

Various fates include the oxidative decarboxylation of malate, forming pyruvate by the NADP-dependent malic enzyme, the dehydration of malate to form fumarate needed as an acceptor of anaerobic electrons via the electron-transport chain coupled fumarate reductase reaction, or the oxidation of malate to oxaloacetate generating the reduced NADH needed to drive the reduction of fumarate (deZwaan, 1977).

The distribution of the malate metabolizing enzymes between the mitochondrial and cytosolic compartments is an important consideration in the understanding of the origin of accumulating endproducts. Succinate and alanine production is mitochondrial, as the electron-transport chain-linked fumarate reductase is located in this cell compartment (Holwerda and deZwaan, 1979, 1980), and the only enzyme activity identified in this organism thus far which can synthesize alanine, the alanine aminotransferase (Paynter et al., 1984a).

Aspartate can also act as a carrier of carbon and nitrogen for alanine and succinate synthesis (Graham and Ellington, 1985). Aspartate breakdown is favored in the mitochondrion yielding oxaloacetate while aspartate synthesis from oxaloacetate seems to be favored in the cytosol of the ribbed mussel (Paynter et al., 1984b).

Some of the kinetic properties of the cMDHs from the molluscs Mytilus edulis and Patella caerulea have been reported (Livingstone, 1976; Lazou et al., 1987); however, to our knowledge there is no previous report in the literature on the properties of the mMDH from any mollusc species. As there are several possible fates of malate and oxaloacetate once inside the mitochondrion, the enzymes constituting the "malate branchpoint" (deZwaan, 1977) within the mitochondria need special consideration. We herein consider the kinetic properties of one of the important enzymes of the mtiochondrial malate branchpoint, the mMDH and also the cMDH from the gill tissue of the ribbed mussel.

MATERIALS AND METHODS

Chemicals and Regents: Malic acid, oxaloacetic acid, NAD (99%), NADH disodium salt and most other reagents were obtained from Sigma (St. Louis), with the following exceptions: CAPS was obtained from P-L Biochemicals (Milwaukee, Wis.); succinic acid disodium salt was obtained from Eastman (Rochester, N.Y.), diethanolamine (purified), magnesium acetate, and magnesium chloride were obtained from Fisher (Fair Lawn, N.J.)

Enzyme purification: mMDH activity was purified as described in the previous chapter (Chapter 1). A highly purified preparation of the least anodally migrating cytosolic isozyme (C1), also described in Chapter 1, was used as the cytosolic form of the enzyme.

Enzyme assay: Assays were performed in duplicate with usually less than 10 % variation between replicates. Reaction mixtures to measure MDH in the OAA utilizing direction were performed in 50 mM HEPES buffer. The concentrations of other assay components are indicated in the figure legends. Reaction mixtures to measure MDH activity in the malate utilizing direction were modified after Smith (1983) and contained the concentrations of diethanolamine (DEA), sodium malate, and NAD indicated in the figure legends. The pK for DEA at 25°C is pH 8.8 (CRC Handbook). Several potential inhibitors (sodium glutamate, $MgCl_2$, and magnesium acetate) were also included in the assay mixtures as indicated.

Aqueous stock solutions of the various substrates were used in these experiments. Free acid solutions (malate, aspartate, glutamate, alanine,

hydroxymalonate, oxaloacetate) were adjusted with the addition of NaOH to the pH of the buffer used in a particular experiment. Oxaloacetate solutions were maintained on ice and used within six hours of preparation.

Kinetic Analysis: The apparent K_m's for NADH and NAD were determined using Cleland's (1979) computer program for hyperbolic kinetics. The apparent K_m's for malate and oxaloacetate were determined using Cleland's (1979) substrate inhibition program. Hydroxymalonate inhibition data were analyzed by Least Squares Analysis, and the reported K_i values were the K_{islope}'s (Segel, 1975). The apparent K_i's for several of the salts and dicarboxylic acids tested for inhibition were determined using the equation

$$I_{50} = (1+[S]/K_m)(K_i).$$

RESULTS

Both the cMDH and mMDH were reactive with a broad range of oxaloacetate concentrations (Fig. 1). The apparent K_m's of the cMDH and the mMDH for oxaloacetate were low at pH 8 (10 μM for the cMDH and 8 μM for the mMDH). The apparent K_m increased when the pH of the assay mixture was increased to 9.2 (105 μM for the cMDH and 47 μM for the mMDH). Both forms showed substrate inhibition at pH 8 and pH 9.2; however substrate inhibition was more pronounced at pH 8. From the substrate inhibition data, both the cMDH and mMDH were about equally inhibited by OAA at pH 8 (K_i's of 4.13 and 2.93 mM for the c and mMDH, respectively). At pH 9.2, the mMDH appeared to be more sensitive to substrate inhibition by OAA (K_i's of 106 and 17.09 mM for the c and mMDH, respectively). Inclusion of 10 mM $MgCl_2$ increased the apparent K_m's for oxaloacetate of both the cMDH and the mMDH (571 μM for the cMDH and 132 μM for the mMDH). The apparent K_i for $MgCl_2$ is low for oxaloacetate utilization by both the c and mMDH (2.34 and 4.25 mM for c and mMDH, respectively). The statistical analysis of these data made with Clelands's (1979) computer program for substrate inhibition is given in Table 1.

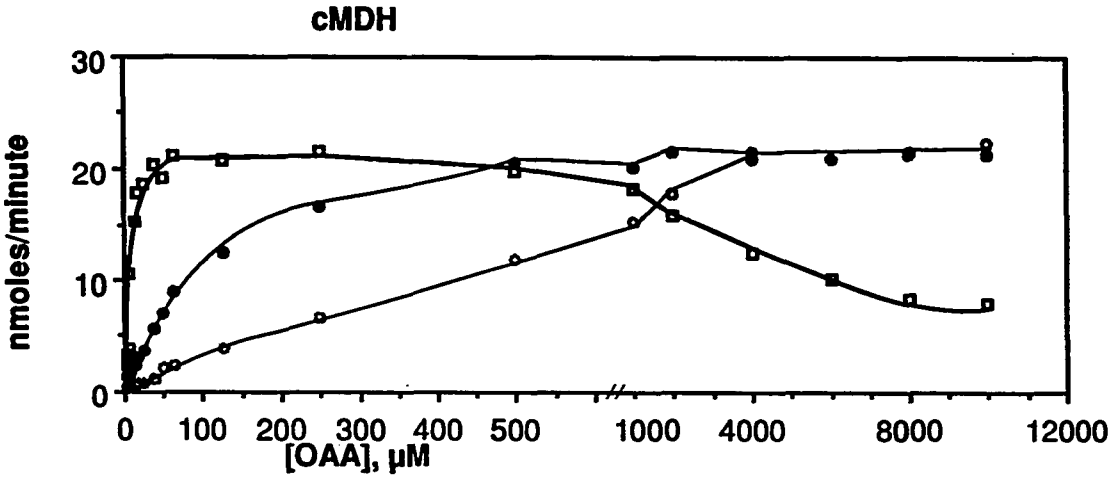
At pH 8, both the cMDH and mMDH were similarly reactive with NADH (Fig. 2). The apparent K_m's for the two forms were 6 μM (cMDH) and 19 μM (mMDH). Increasing the pH of the reaction mixture to pH 9.2 increased the apparent K_m's of both forms slightly (17 μM for the cMDH and 35 μM for the mMDH). Including $MgCl_2$ in the reaction mixture increased the apparent K_m for NADH of the cMDH, but had little effect on the apparent K_m for NADH of

the mMDH. The apparent K_i's for $MgCl_2$ for NADH utilization for the c and mMDH are considerably higher than observed for oxaloacetate utilization (9.42 and 34.10 mM for the c and mMDH, respectively). These data are summarized in Table 1.

As measurement of the malate utilizing direction was difficult at pH 8 due to the non-linearity of the recordings, measurement of this direction was made only at pH 9.2. The cMDH had a lower apparent K_m for malate than the mMDH (464 μM for the cMDH and 1,060 μM for the mMDH). From the data shown in Fig. 3a, it appeared that the inclusion of $MgCl_2$ had little effect on malate binding to the cMDH while it had the effect of increasing the apparent K_m for the mMDH. However, a statistical analysis of these data showed that both forms are inhibited by $MgCl_2$ (Table 1). The apparent K_i's for $MgCl_2$ inhibiton of malate utilization were considerably higher than those observed for oxaloacetate utilization for both the c and mMDHs (14.84 mM and 16.41 mM for the c and m MDH, respectively). Both the c and mMDHs were inhibited by high concentrations of malate. From the plot of activity vs. malate concentration, it appeared that the cMDH was more sensitive to substrate inhibtion by malate; however, analysis of these data with Cleland's computer program indicated that the cMDH had a higher K_i for malate than the mMDH (257 vs. 141 mM) and is therefore less sensitive to substrate inhibition.

An investigation of the effects of $MgCl_2$ and Mg acetate over a limited range of malate concentrations (Figure 3b) indicated that these compounds were competitive inhibitors with apparent K_i's of the order calculated for

Figure 1. OAA Utilization by the cMDH and mMDH from the Gill Tissue of the Ribbed Mussel. Reaction mixtures contained enzyme, the indicated quantities of OAA and 150 μM NADH in 50 mM HEPES pH 8.0 (open squares), or with 100 mM diethanolamine pH 9.2 (closed circles), or with 100 mM diethanolamine pH 9.2 plus 10 mM $MgCl_2$ (open circles).


cMDH
nmoles/minute
30
20
10
0
0
100
200
300
400
500
1000
4000
8000
12000
[OAA], µM

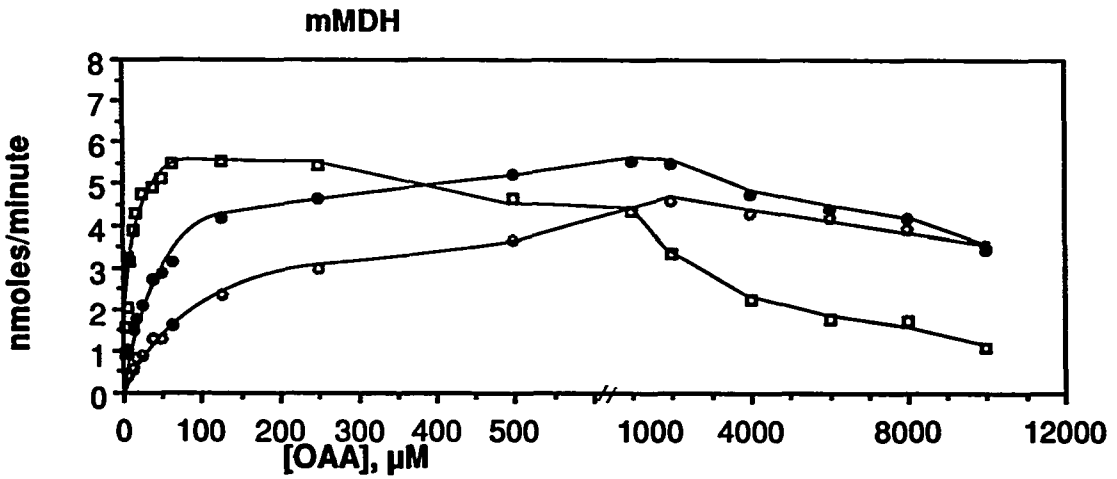
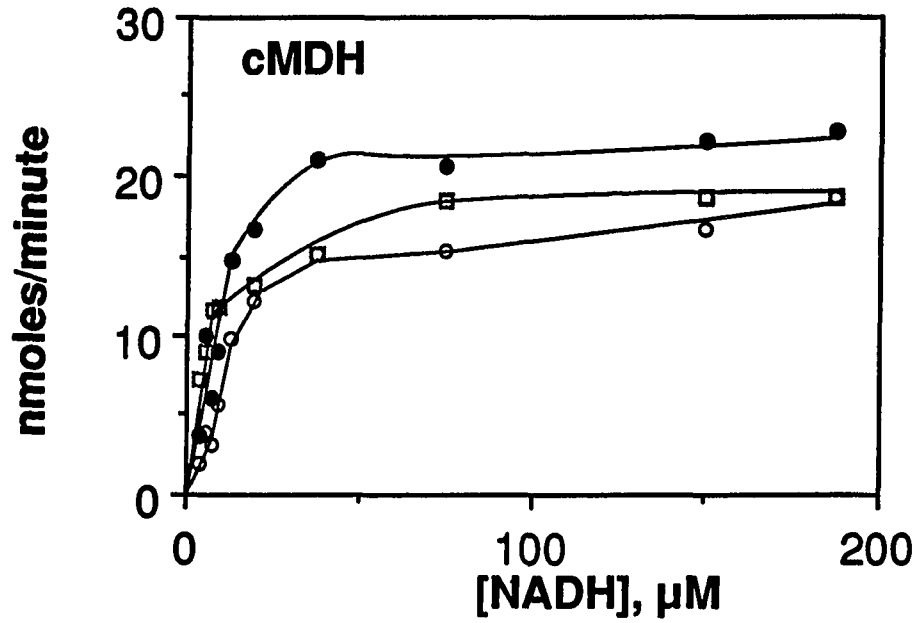

mMDH
nmoles/minute
8
7
6
5
4
3
2
1
0
0
100
200
300
400
500
1000
4000
8000
12000
[OAA], µM

Figure 2. NADH Utilization by the cMDH and mMDH from the Gill Tissue of the Ribbed Mussel. Reaction mixtures contained enzyme, the indicated quantities of NADH, and 500 μM OAA in 50 mM HEPES pH 8.0 (open squares), or with 100 mM diethanolamine pH 9.2 (closed circles), or 100 mM diethanolamine pH 9.2 plus 10 mM $MgCl_2$ (open circles).


cMDH
nmoles/minute
30
20
10
0
0
100
200
[NADH], µM

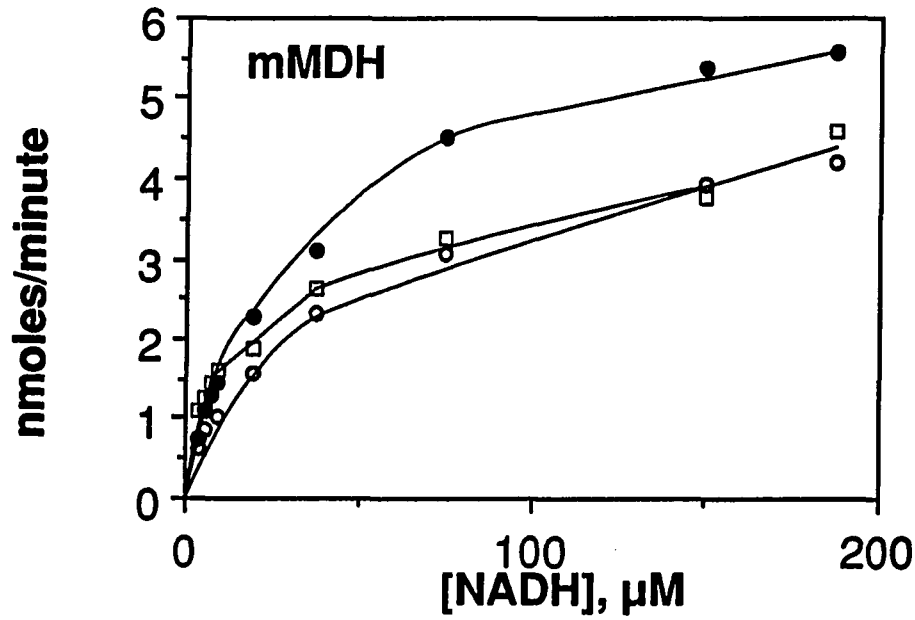
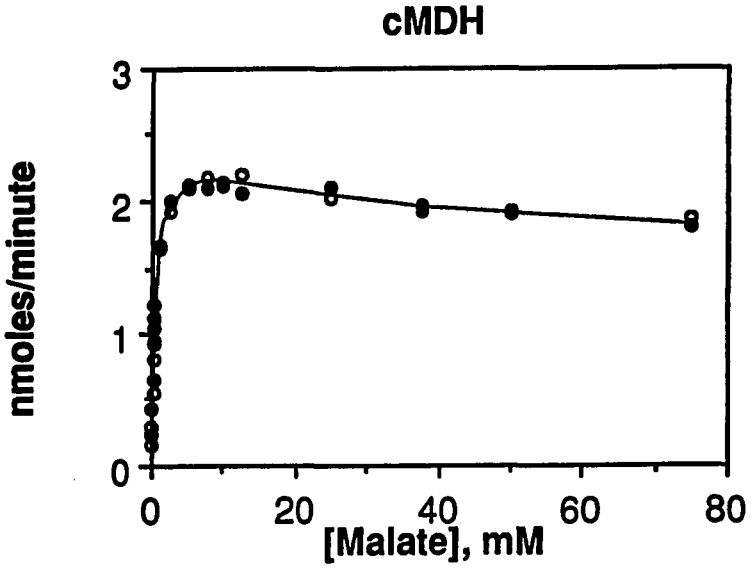

mMDH
nmoles/minute
6
5
4
3
2
1
0
0
100
200
[NADH], µM

Figure 3a. Malate utilization by the cMDH and mMDH of the Gill Tissue of the Ribbed Mussel. Reaction mixtures contained enzyme, the indicated quantities of sodium malate and 400 μM NAD in 100 mM diethanolamine pH 9.2 (closed circles) or 100 mM diethanolamine plus 10 mM $MgCl_2$ (open circles).


cMDH
nmoles/minute
3
2
1
0
0
20
40
60
80
[Malate], mM

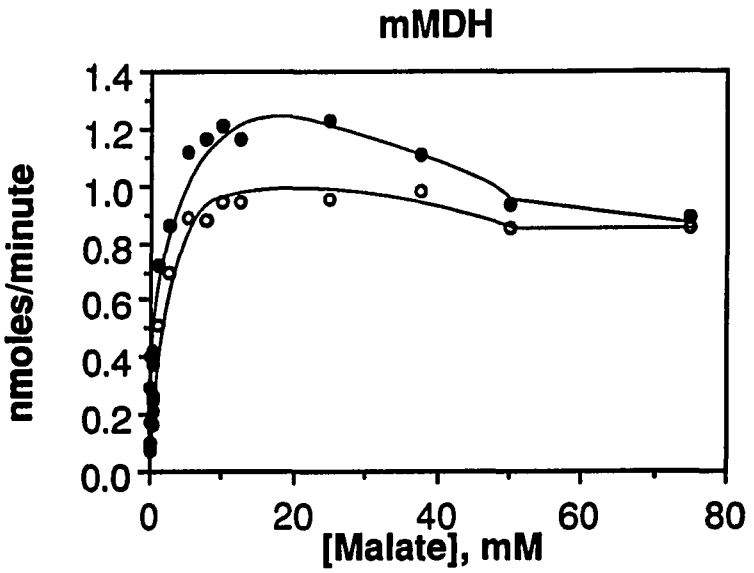
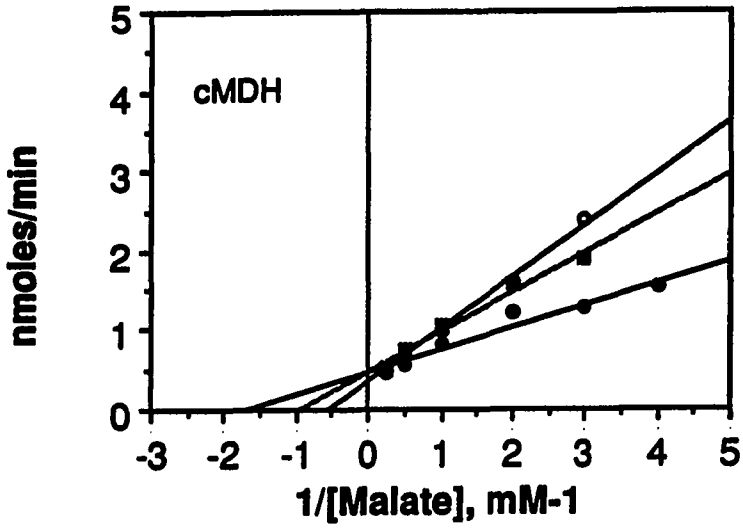

mMDH
nmoles/minute
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
[Malate], mM

Figure 3b. Effect of $MgCl_2$ and Mg Acetate on Malate Utilization by the cMDH and mMDH. Reaction mixtures contained enzyme, 100 mM DEA (pH 9.2), 200 μM NAD, and the indicated concentrations of malate. Closed circles: controls. Open circles: With 10 mM $MgCl_2$. Open squares: With 10 mM Mg Acetate.


cMDH
nmoles/min
1/[Malate], mM-1
5
4
3
2
1
0
-3
-2
-1
0
1
2
3
4
5

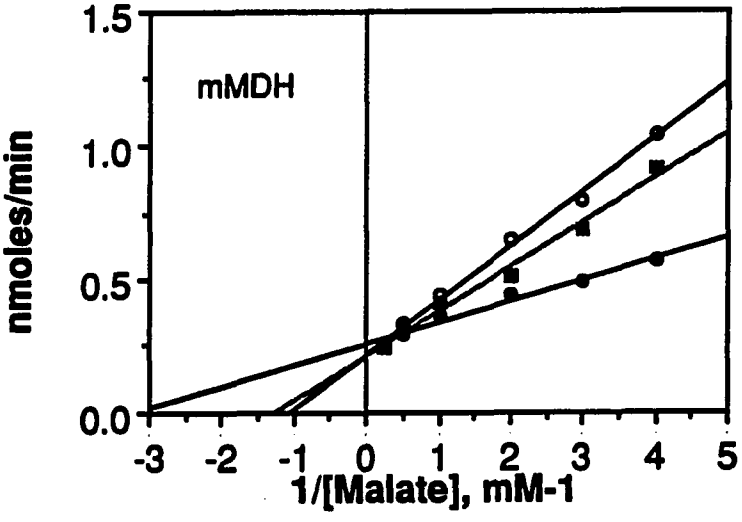
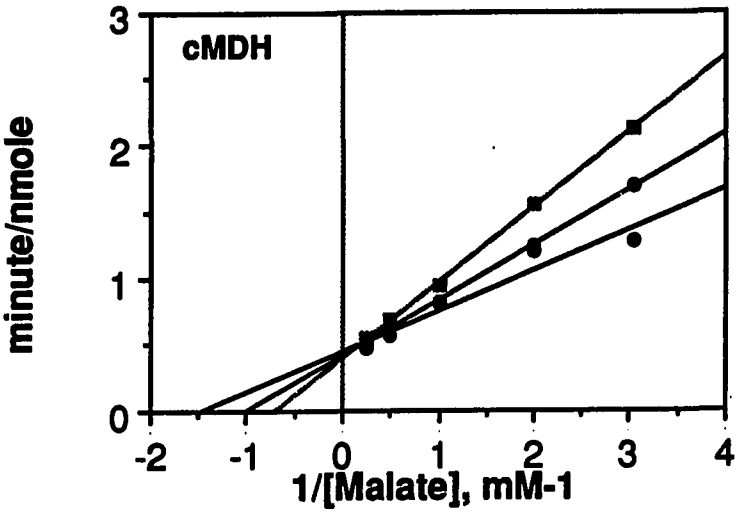

mMDH
nmoles/min
1/[Malate], mM-1
1.5
1.0
0.5
0.0
-3
-2
-1
0
1
2
3
4
5

Figure 3c. Effect of Glutamate and $MgCl_2$ Inclusion on Malate Utilization by the cMDH and mMDH. Reaction mixtures contained enzyme, 100 mM DEA (pH 9.2), 200 μM NAD, and the indicated concentrations of malate. Closed circles: controls. Open circles: plus 10 mM sodium glutamate. Closed square: plus 10 mM sodium glutamate and 10 mM $MgCl_2$.


cMDH
minute/nmole
3
2
1
0
-2
-1
0
1
2
3
4
1/[Malate], mM-1

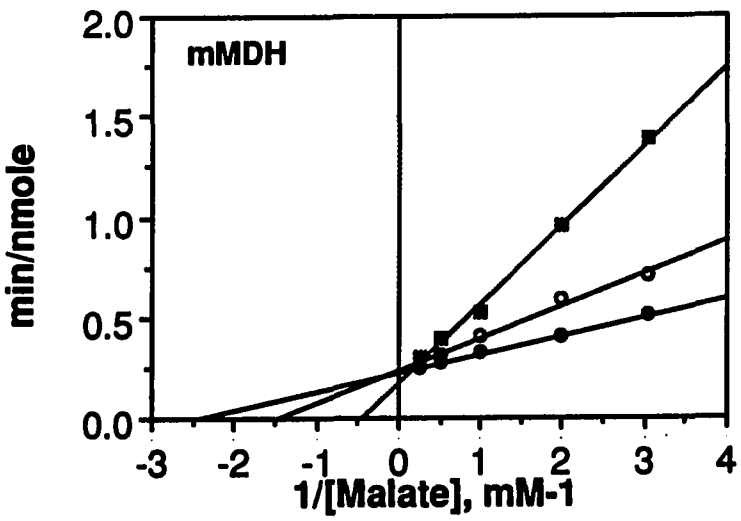
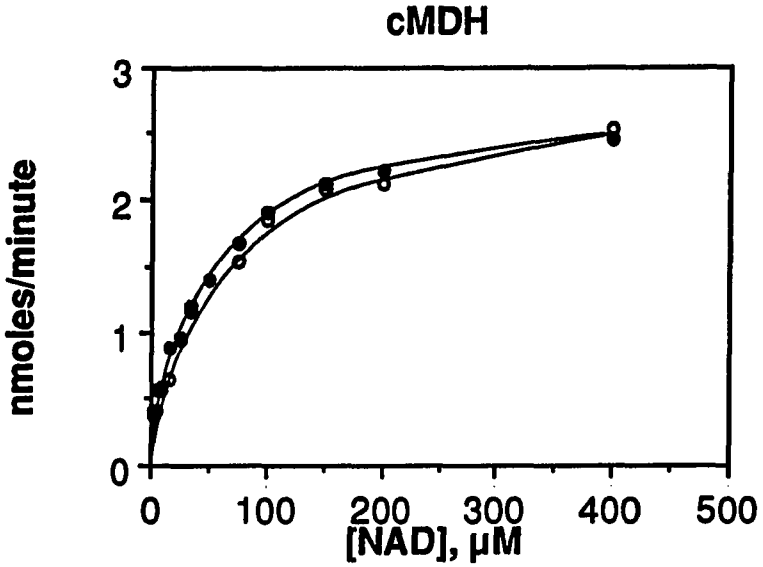

mMDH
min/nmole
2.0
1.5
1.0
0.5
0.0
-3
-2
-1
0
1
2
3
4
1/[Malate], mM-1

Figure 4. NAD Utilization by the cMDH and mMDH of the Gill Tissue of the Ribbed Mussel. Reaction mixtures contained enzyme, the indicated quantities of NAD and 10 mM sodium malate in 100 mM diethanolamine pH 9.2 (closed circles) or 100 mM diethanolamine plus 10 mM $MgCl_2$ (open circles).


cMDH
nmoles/minute
3
2
1
0
0
100
200
300
400
500
[NAD], µM

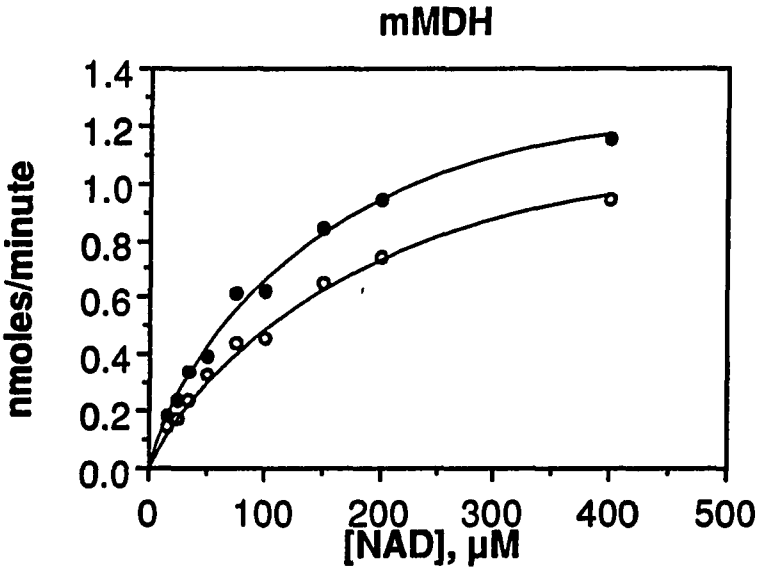

mMDH
nmoles/minute
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
[NAD], µM

Table 1. Summary of c and m MDH Kinetic Constants[a]

cMDH			
Substrate	pH 8	pH 9.2	pH 9.2+$MgCl_2$
OAA: K_m	10.26±1.91 µM[b]	104.81±7.84 µM	571.30±51.28 µM
OAA: V_{max}	24.22±1.20 nmoles/min	23.16±0.52 nmoles/min	23.95±0.89 nmoles/min
OAA: K_i	4.13±0.79 mM	106.36±48.18 mM	332.65mM±528.44 mM
$MgCl_2$ K_{islope}	----	----	2.34 mM
Malate: K_m	----	464.18±18.15 µM	830.74±211.39 µM
Malate: V_{max}	----	2.31±0.03 nmoles/min	2.47±0.20 nmoles/min
Malate: K_i	----	257.40±24.90 mM	189.78±103.38 mM
$MgCl_2$ K_{islope}	----	----	14.84 mM
NADH: K_m	6.10±0.71 µM	17.27±1.71 µM	29.75±5.91 µM
NADH: V_{max}	19.00±0.53 nmoles/min	24.77±0.70 nmoles/min	20.62±1.31 nmoles/min
$MgCl_2$ K_{islope}	----	----	9.42 mM
NAD: K_m	----	37.88±5.58 µM	44.54±5.50 µM
NAD:V_{max}	----	2.62±0.12 nmoles/min	2.67±0.11 nmoles/min
$MgCl_2$ K_{islope}	----	----	65.14 mM

mMDH

OAA: K_m	8.23±1.40 µM	47.15±4.32 µM	132.42±13.40 µM
OAA: V_{max}	6.06±0.27 nmoles/min	5.93±0.17 nmoles/min	4.97±0.18 nmoles/min
OAA: K_i	2.93±0.47 mM	17.09±2.50 mM	30.37±6.92 mM
$MgCl_2$ Ki_{slope}	----	----	4.25 mM
Malate: K_m	----	1,061.52±135.68 µM	1,351.85±108.36 µM
Malate: V_{max}	----	1.39±0.05 nmoles/min	1.10±0.03 nmoles/min
Malate: K_i	----	141.65±30.25 mM	261.56±50.04 mM
$MgCl_2$ Ki_{slope}	----	----	16.41 mM
NADH: K_m	19.39±4.45 µM	35.01±2.87 µM	34.83±10.55 µM
NADH: V_{max}	4.46±0.31 nmoles/min	6.58±0.18 nmoles/min	5.06±0.56 nmoles/min
$MgCl_2$ Ki_{slope}	----	----	34.10 mM
NAD: K_m	----	150.50±14.43 µM	189.15±18.60 µM
NAD: V_{max}	----	1.67±0.07 nmoles/min	1.45±0.06 nmoles/min
$MgCl_2$ Ki_{slope}	----	----	22.35 mM

[a]Kinetic constants were determined using Cleland's (1979) computer programs.

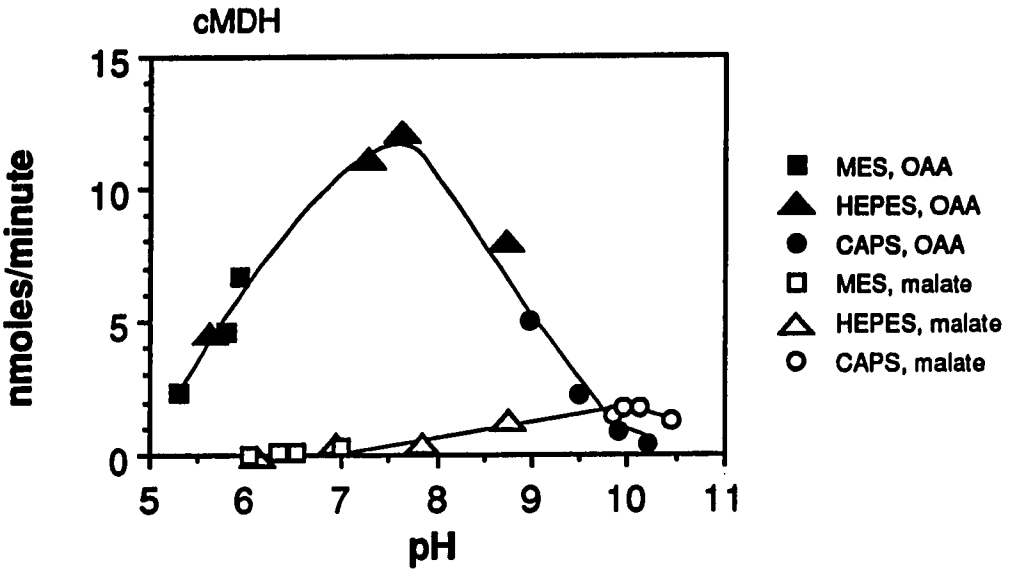
[b]Values are constants plus or minus standard errors.

$MgCl_2$ over the broad range of malate concentrations shown in Figure 3a and summarized in Table 1. For the cMDH, the K_i for $MgCl_2$ was 8.82 mM while the K_i for Mg acetate was 16.07 mM. For the mMDH, the K_i for $MgCl_2$ under these conditions was 8.05 mM while the K_i for Mg acetate was 11.24 mM. The comparable values for $MgCl_2$ reported in Table 1 were 14.84 mM for the cMDH and 16.41 mM for the mMDH. The differences in the K_i values for $MgCl_2$ under the two experimental conditions may be due to the different statistical analysis used (Cleland's method vs. least squares analysis) and possibly to differences in the range of concentrations chosen.

Glutamate was a competitive inhibitor of malate utilization by both the cMDH and mMDH (Figure 3b) and addition of 10 mM $MgCl_2$ enhanced this effect. For the mMDH, the calculated slope of the double reciprocal plot in the absence of inhibitor was 0.091. When 10 mM glutamate was added, the slope increased to 0.181, indicating an apparent K_i for glutamate of 10.11 mM. When 10 mM $MgCl_2$ was added in the presence of 10 mM sodium glutamate. the slope increased to 0.399. For the cMDH, the calculated slope of the double reciprocal plot in the absence of inhibitor was 0.307. When 10 mM sodium glutamate was added, the slope increased to 0.412, indicating an apparent K_i for glutamate of 29.24 mM. When 10 mM $MgCl_2$ was added in the presence of 10 mM sodium glutamate, the slope increased to 0.566. The additive competitive effect of these inhibitors suggested that they both interfered with the dicarbxylic acid binding site.

The cMDH had a lower apparent K_m for NAD than the mMDH (38 μM for the cMDH and 151 μM for the mMDH). The mMDH appeared to be more

Figure 5. Effect of pH on the Activity of the Cytosolic Form and the Mitochondrial Form of the MDH's from the Ribbed Mussel Gill. Reaction rates in the oxaloacetate using direction were performed in reaction mixtures containing enzyme, 50 mM of the indicated buffer at the indicated pH with 200 μM oxaloacetate and 215 μM NADH. Reaction rates in the malate using direction were performed in reaction mixtures containing enzyme, 50 mM of the indicated buffer at the indicated pH, 10 mM malate, and 200 μM NAD.


cMDH
nmoles/minute
15
10
5
0
5
6
7
8
9
10
11
pH
MES, OAA
HEPES, OAA
CAPS, OAA
MES, malate
HEPES, malate
CAPS, malate

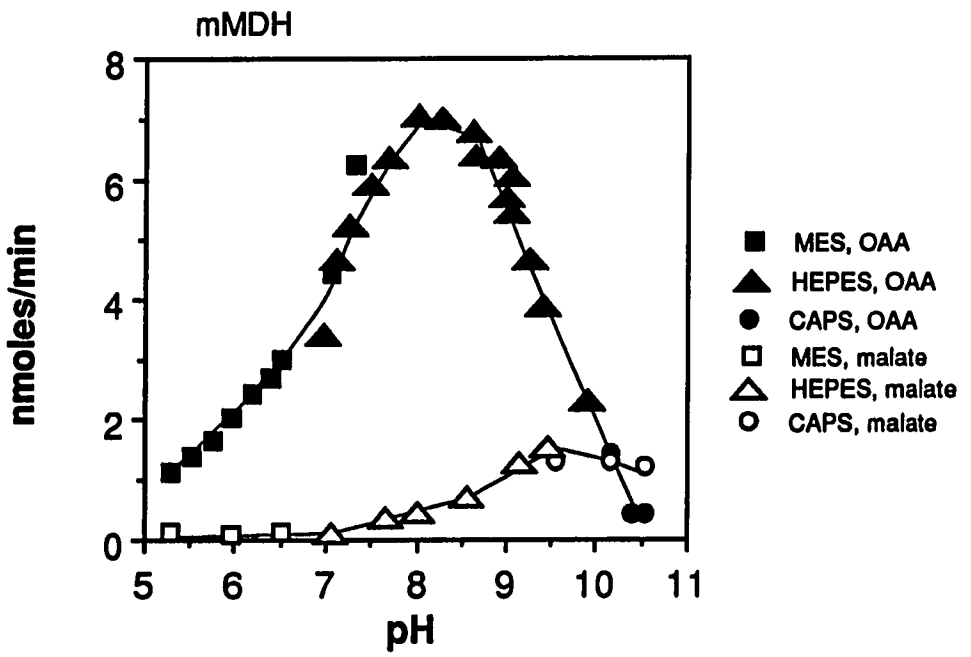
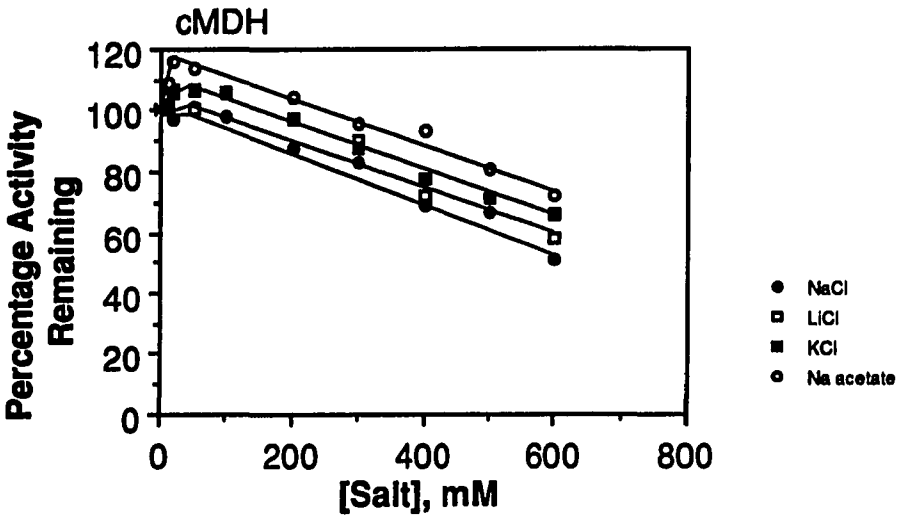

mMDH
nmoles/min
8
6
4
2
0
5
6
7
8
9
10
11
pH
MES, OAA
HEPES, OAA
CAPS, OAA
MES, malate
HEPES, malate
CAPS, malate

Figure 6. Inhibition of the Cytosolic and Mitochondrial Malate Dehydrogenases by Several Salts. Reaction mixtures contained enzyme, 50 mM HEPES (pH 8), 200 μM oxaloacetate, 215 μM NADH, and the indicated concentrations of the various salts.


cMDH
Percentage Activity Remaining
120
100
80
60
40
20
0
0
200
400
600
800
[Salt], mM
NaCl
LiCl
KCl
Na acetate

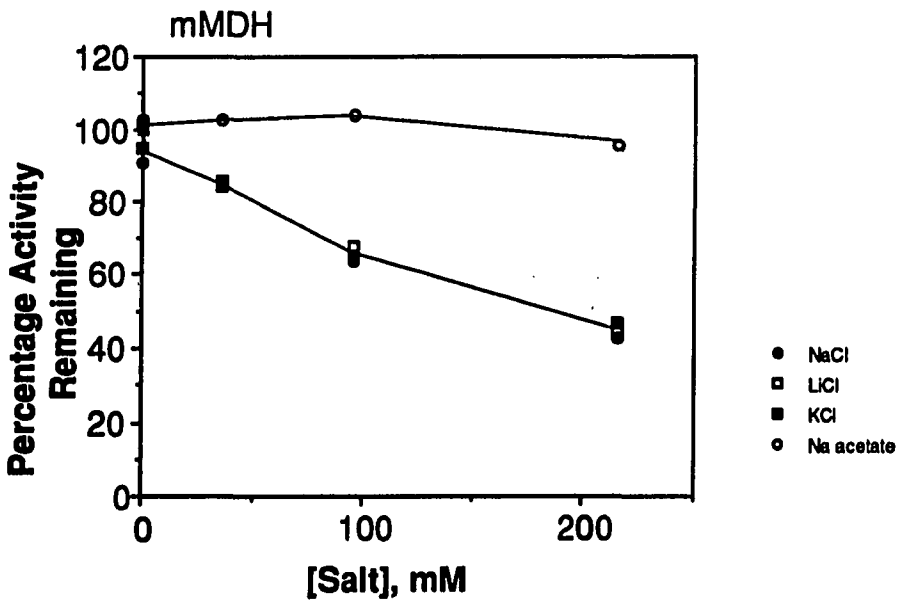
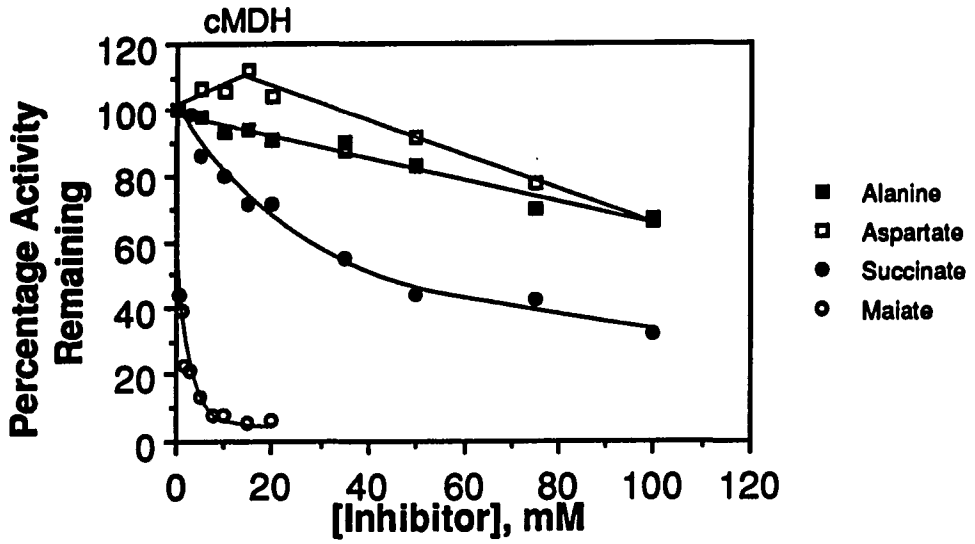

mMDH
Percentage Activity Remaining
120
100
80
60
40
20
0
0
100
200
[Salt], mM
NaCl
LiCl
KCl
Na acetate

Figure 7. Inhibition of the Cytosolic and Mitochondrial MDHs by Several Dicarboxylic Acids and Alanine. Reaction mixtures contained enzyme, 50 mM HEPES (pH 8), 20 μM oxaloacetate, 215 μM NADH, and the various indicated concentrations of inhibitors.


cMDH
Percentage Activity Remaining
120
100
80
60
40
20
0
0
20
40
60
80
100
120
[Inhibitor], mM
Alanine
Aspartate
Succinate
Malate

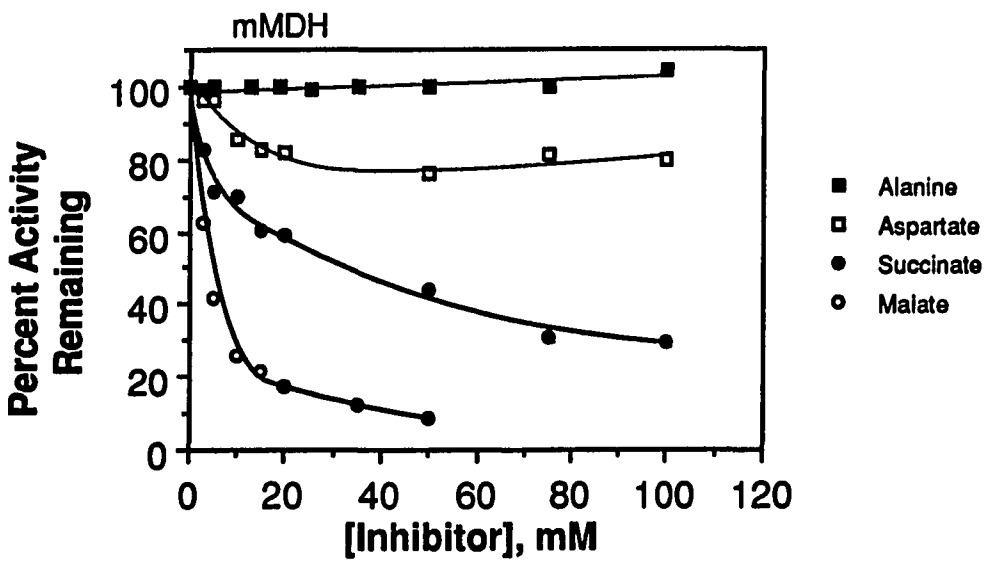

mMDH
Percent Activity Remaining
100
80
60
40
20
0
0
20
40
60
80
100
120
[Inhibitor], mM
Alanine
Aspartate
Succinate
Malate

Table 2. Apparent K_i's for Several Dicarboxylic Acids and Salts [a]

inhibitor	cMDH I_{50};	$K_i$app.	mMDH I_{50};	$K_i$app.
malate	0.5 mM;	0.16 mM	2 mM;	0.60 mM
succinate	35 mM;	12 mM	42 mM;	12 mM
NaCl	600 mM;	29 mM	160 mM;	6.2 mM
KCl	650 mM;	31 mM	165 mM;	6.3 mM
LiCl	700 mM;	33 mM	170 mM;	6.5 mM
sodium acetate	750 mM;	36 mM	---------[b]	---------

[a]Conditions of assays are described in legends for Figures 6 and 7.

[b]Inhibition below detectable limits (no inhibition).

sensitive to inhibition by $MgCl_2$ than the cMDH (Figure 4), but a statistical analysis of these data showed that both forms are slightly inhibited by $MgCl_2$ inclusion (Table 1). From these data, the apparent K_i's of $MgCl_2$ for NAD utilization were quite high (65.14 and 22.35 mM for the c and mMDH, respectively).

The pH optima for the OAA and malate utilizing directions of the cMDH and the mMDH were determined over a broad range using three of Good's buffers (MES, HEPES, and CAPS). The mMDH pH optimum for OAA utilization was found to be slightly higher than the pH optimum of the cMDH (8.5 for the mMDH and 7.5 for the cMDH), while both forms had similar pH optima for malate utilization of about pH 9.5-10 (Fig. 5).

Under identical conditions, the cMDH was much less sensitive to inhibition by the chloride derivatives of sodium, potassium, and lithium than the mMDH. While the mMDH was less sensitive to inhibition by sodium acetate than by the other salts tested, the cMDH was about equally sensitive to inhibition by sodium acetate as by the other salts tested (Fig. 6). The apparent K_i values for the several salts tested are presented in Table 2.

Each of the dicarboxylic acids tested inhibited the oxaloacetate utilizing activity of both the MDHs (Fig. 7). Malate was most inhibitory, followed by succinate and then by aspartate. Alanine inhibited the cMDH to a small degree and had no effect on the mMDH.

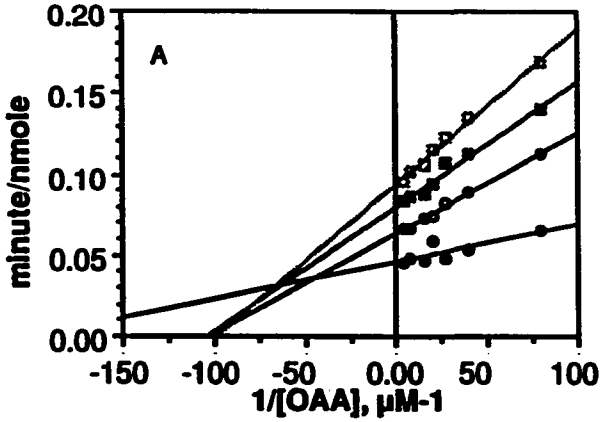
Figure 8. Hydroxymalonate Inhibits the cMDH and mMDH of the Gill Tissue of the Ribbed Mussel. Reaction mixtures contained 50 mM HEPES (pH8), 150 μM NADH, the indicated quantities of OAA, and either 0 mM hydroxymalonate (closed circle), 1 mM hydroxymalonate (open circle), 2 mM hydroxymalonate (closed square), or 3 mM hydroxymalonate (open square).

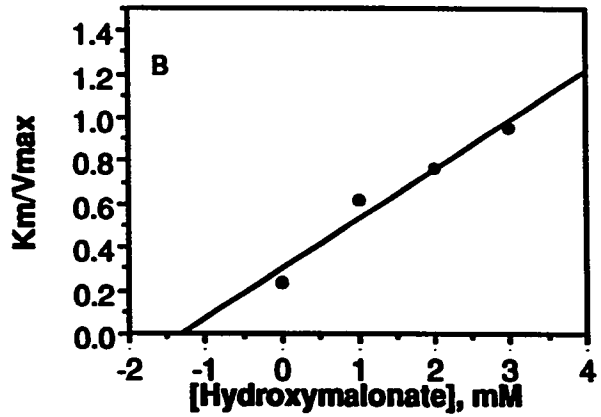
Part A: Lineweaver-Burk Plot of Influence of Hydroxymalonate on the cMDH's Reactivity with Oxaloacetate.

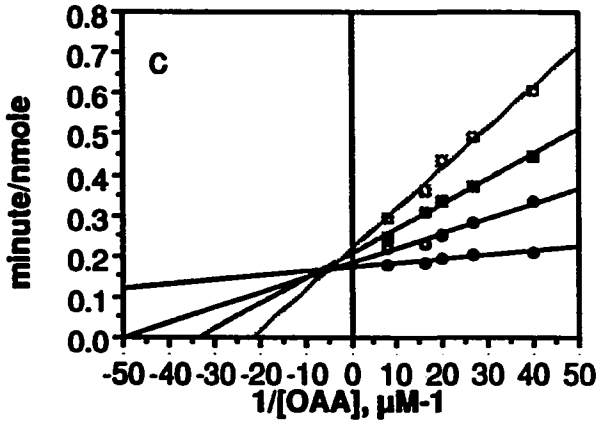
Part B: Replot of Slope from Part A as a Function of Hydroxymalonate Concentration.

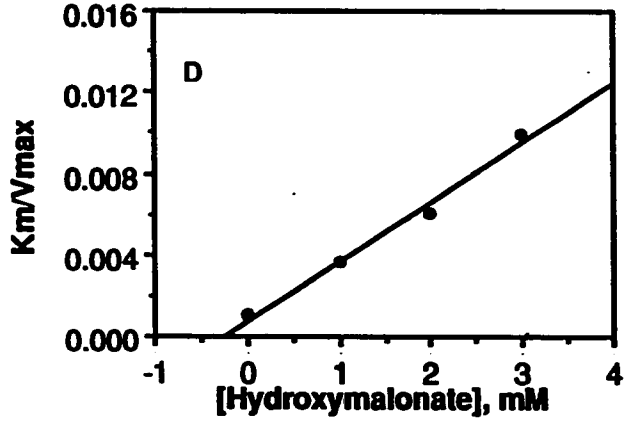
Part C: Lineweaver-Burk Plot of Influence of Hydroxymalonate on the mMDH's Reactivity with Oxaloacetate.

Part D: Replot of Slope from Part C as a Function of Hydroxymalonate Concentration.


A
minute/nmole
0.20
0.15
0.10
0.05
0.00
-150 -100 -50 0.00 50 100
1/[OAA], µM-1


B
Km/Vmax
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-2 -1 0 1 2 3 4
[Hydroxymalonate], mM


C
minute/nmole
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-50 -40 -30 -20 -10 0 10 20 30 40 50
1/[OAA], µM-1


D
Km/Vmax
0.016
0.012
0.008
0.004
0.000
-1 0 1 2 3 4
[Hydroxymalonate], mM

From the data presented in Figure 7, apparent K_i values were calculated for several of the acids tested (Table 2). For the cMDH, the apparent K_i's for the salts sodium chloride, potassium chloride, lithium chloride, and sodium acetate ranged between 29 and 36 mM. For the mMDH, the apparent K_i's for sodium chloride, potassium chloride, and lithium chloride ranged between 6.2 and 6.5 mM. This analysis also showed that the two forms bound succinate about equally, while the cMDH bound malate more effectively than the mMDH. By comparison of the malate apparent K_i's at pH 8 with the apparent K_m's at pH 9.2, it appeared that increasing the pH of the assay mixture decreased the affinity of both enzymes for malate. Specifically, the apparent K_m of the cMDH for malate at pH 9.2 was 0.5 mM, whereas the apparent K_i at pH 8 was 0.16 mM. The apparent K_m of the mMDH at pH 9.2 for malate was 1 mM whereas the apparent K_i at pH 8 was 0.60 mM (Table 2).

Both forms were inhibited by millimolar concentrations of the malate analogue hydroxymalonate when assayed in the OAA utilizing direction (Fig. 8). While the pattern of inhibition of the mMDH was competitive, hydroxymalonate inhibited the cMDH in a mixed non-competitive fashion. The K_{islope}'s for hydroxymalonate of the two forms were 0.256 mM for the mMDH and 1.17 mM for the cMDH.

NAD was a competitive inhibitor of the cMDH and mMDH from the ribbed mussel with apparent K_i's of 0.60 mM and 0.82 mM for the cMDH and mMDH, respectively (Fig. 9). These values are considerably larger than the apparent K_m's observed for NAD at pH 9.2 (38 μM and 150 μM for the cMDH and mMDH, respectively).

ATP was a competitive inhibitor of NADH binding by the cMDH but was a mixed inhibitor of NADH binding by the mMDH (Fig. 10). Based on all three of the ATP concentrations used in the cMDH experiment, the cMDH had an apparent K_i for ATP of 0.77 mM. ATP inhibition of NAD by the mMDH was initially attempted over the same range of concentrations as the cMDH, but a non-linear slope vs ATP concentration replot was immediately apparent. Therefore, ATP inhibition was examined over a broader range of ATP concentrations for this enzyme. When a replot of slope vs. ATP concentration was made, linearity was observed when the zero ATP point was omited. By linear regression, the apparent K_i for ATP of the mMDH was 5.38 mM (Figure 10). The hydroxymalonate and ATP inhibition data are summarized in Table 3.

Figure 9. NAD is a Competitive Inhibitor of NADH for the Cytosolic and Mitochondrial Malate Dehydrogenases from the Ribbed Mussel Gill. Reaction mixtures contained enzyme, 50mM HEPES (pH 8),200 μM oxaloacetate, and the indicated concentrations of NADH. Closed circles: 0 NAD. Open circles: 1 mM NAD. Closed squares: 2 mM NAD.

Part A: Effect of NAD on NADH reactivity of the cMDH.

Part B: Slopes from Part A as a Function of NAD Concentration.

Part C: Effect of NAD on NADH reactivity of the mMDH.

Part D: Slopes from Part C as a Function of NAD Concentration.

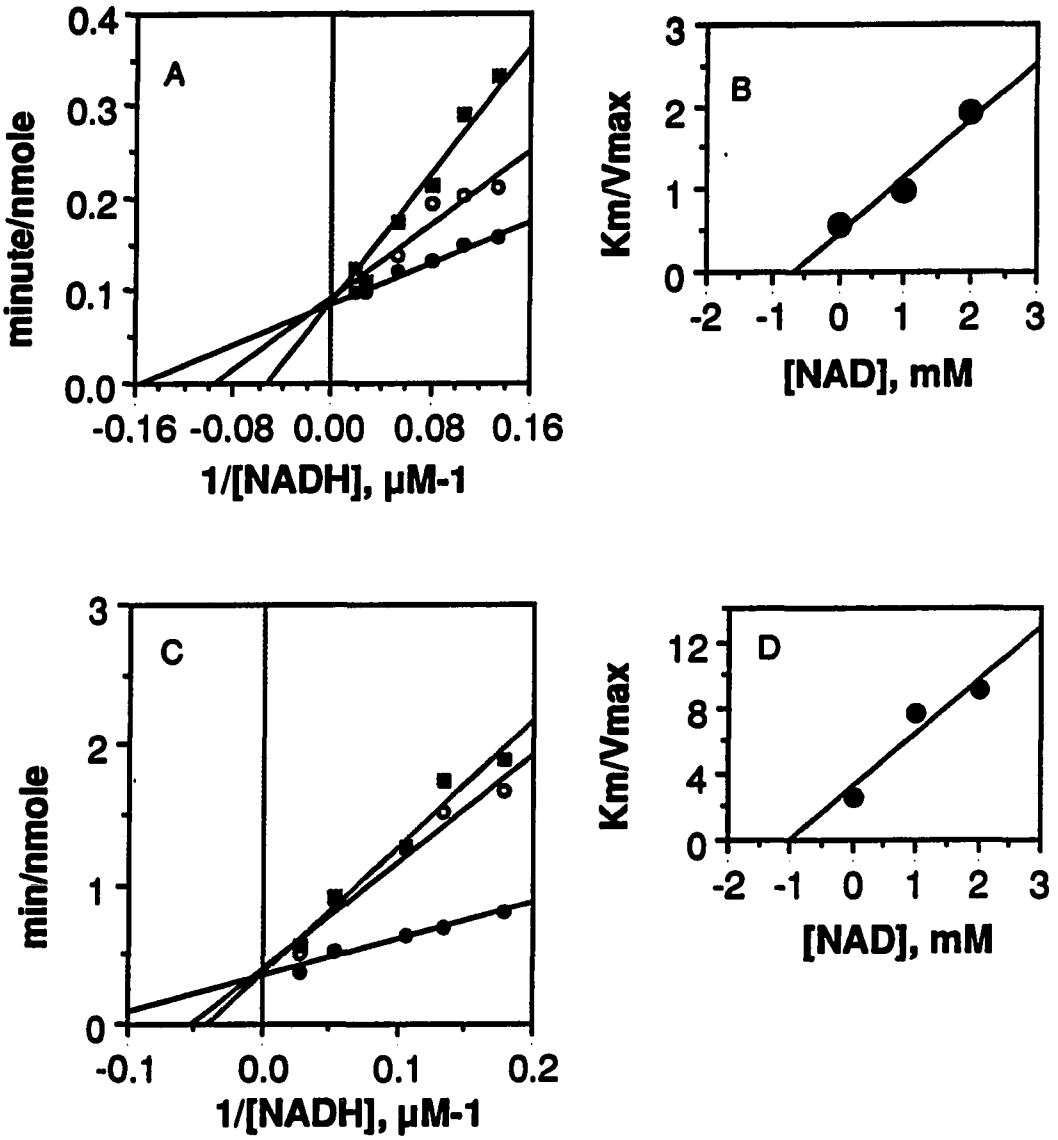

A
minute/nmole
0.4
0.3
0.2
0.1
0.0
-0.16 -0.08 0.00 0.08 0.16
1/[NADH], µM-1
B
Km/Vmax
3
2
1
0
-2 -1 0 1 2 3
[NAD], mM
C
min/nmole
3
2
1
0
-0.1 0.0 0.1 0.2
1/[NADH], µM-1
D
Km/Vmax
12
8
4
0
-2 -1 0 1 2 3
[NAD], mM

Figure 10. ATP Inhibits NADH Binding by the cMDH and mMDH. Reaction mixtures contained enzyme, 50 mM HEPES (pH 8), 200 μM OAA, and the indicated concentrations of NADH.

Part A: Effect of ATP on NADH Reactivity of the cMDH. Closed circles: 0 mM ATP. Open circles: 1 mM ATP. Closed squares: 2 mM ATP.

Part B: Replot of Slopes Calculated from Part A as a Function of ATP Concentration.

Part C: Effect of ATP on NADH Reactivity of the mMDH. Closed circles: 0 mM ATP. Open circles: 1 mM ATP. Closed squares: 2 mM ATP. Open squares: 4 mM ATP. Closed triangles: 6 mM ATP.

Part D: Replot of Slopes Calculated from Part C as a Function of ATP Concentration.

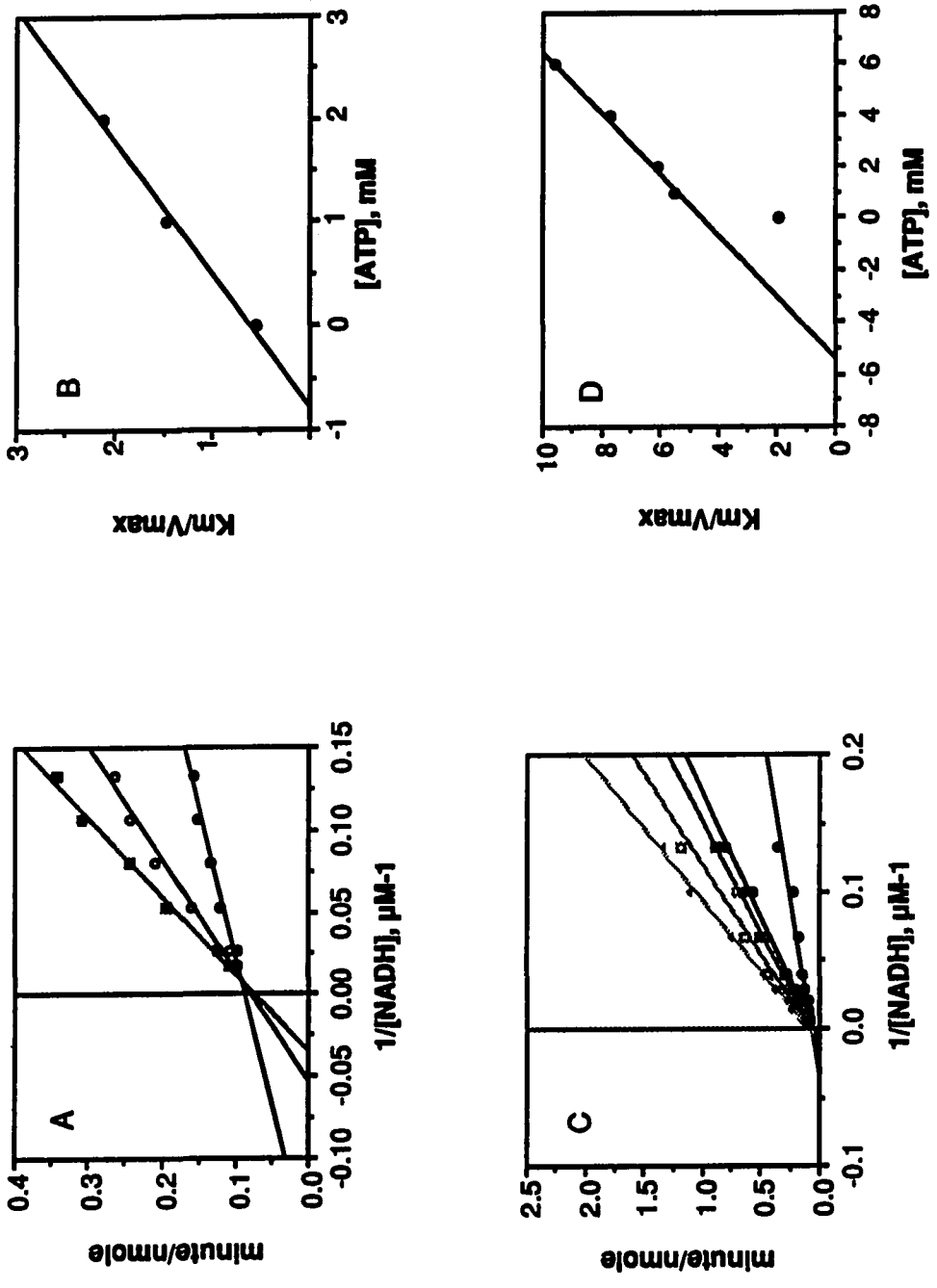

A
minute/nmole
1/[NADH], µM-1
B
Km/Vmax
[ATP], mM
C
minute/nmole
1/[NADH], µM-1
D
Km/Vmax
[ATP], mM

Table 3. Summary of Apparent K_i's for Hydroxymalonate and ATP.

cMDH

Substrate	Inhibitor	Pattern	Apparent K_i
OAA	Hydroxymalonate	Non-Competitive	1.17 mM
NADH	ATP	Competitive	0.77 mM

mMDH

Substrate	Inhibitor	Pattern	Apparent K_i
OAA	Hydroxymalonate	Competitive	0.256 mM
NADH	ATP	Non-Competitive	5.38 mM

DISCUSSION

Substrate inhibition by the dicarboxylic acid substrates is observed with both the cMDH and mMDH. Substrate inhibition by high concentrations of substrate has been reported for many dehydrogenases (Winer and Schwert, 1958; Theorell and McKinley-McKee, 1961; Corman and Kaplan, 1967). While substrate inhibition by oxaloacetate is observed for both the cytosolic and the mitochondrial forms, the mitochondrial form appears to be more sensitive (Fig. 1), consistent with results obtained for the pig heart enzymes (Bernstein et al., 1978) and the chicken heart enzymes (Kitto and Kaplan, 1966). No inhibition by high levels (up to 300 μM) of oxaloacetate is observed for the cMDH form from Patella caerulea (Lazou et al., 1987). For the cMDH from Artemia, no substrate inhibition by oxaloacetate is observed below 7 mM (Hand et al., 1981). Substrate inhibition by malate of the cMDH from several sources is observed (Ozaki and Whiteley, 1970; Hand et al., 1981), and is suggested to be important in the regulation of the oxidation state of cytosolic pyridine nucleotides (Kaplan, 1972). Both the cMDH and mMDH from M. demissus exhibit substrate inhibition at high concentrations of malate. This may suggest regulation of malate oxidation by the MDHs of both compartments.

The apparent K_m's observed for oxaloacetate at pH 8 for the c and m MDHs (Table 1) are considerably lower than values reported for malate dehydrogenases from other sources . For example, the cMDH from Patella caerulea has an apparent K_m for oxaloacetate is 55 μM at pH 7.6 (Lazou et al., 1987). The K_m for oxaloacetate of the cMDH from posterior adductor of M.

edulis is 23 μM. This K_m for oxaloacetate of the cMDH from the mantle of this organism is found to vary over the year, and therefore with the breeding condition of the animal with the K_m measured at 47 μM in May and 135 μM in November (Livingstone, 1976). The K_m for oxaloacetate of the Artemia nauplii cMDH is 42 μM (Hand et al., 1981). The MDHs associated with each of the cell compartments of plants has also been described. For example, the cactus (Opuntia sp.) MDHs associated with the chloroplastic, mitochondrial and soluble compartments have apparent K_m's at pH 7.4 for oxaloacetate of 43, 57 and 76 μM, respectively. With Opuntia, the apparent K_m's for malate at pH 8.5 are 0.97, 1.3, and 1.4 mM, respectively (Mukerji and Ting, 1969). For the spinach malate dehydrogenases, apparent K_m's for oxaloacetate at pH 7.5 of 39, 41, and 58 μM are observed for the microbody, mitochondrial, and soluble malate dehydrogenases, respectively. At pH 8.5, the apparent K_m's for malate for malate dehydrogenases from spinach are 2.84, 5.9, and 0.77 mM for the microbody, mitochondrial, and soluble forms, respectively (Rocha and Ting, 1971).

The apparent K_m for malate of the cMDH from the ribbed mussel at pH 9.2 is comparable to the apparent K_m for the P. caerulea enzyme at pH 7.6 (0.46 mM vs. 0.37 mM). The apparent K_m for the mMDH from M. demissus at pH 9.2 is slightly larger than these values (1.06 mM) However, when the K_m for malate for the mMDH is determined over a narrower concentration range and analysed with least squares analysis, an apparent K_m is determined which is similar to that observed for the cMDHs from these molluscan sources (approximately 0.3 mM; see Figs. 3b and 3c). Therefore, the cMDH and mMDH may have similar affinity for malate. The apparent K_i determined

for these forms by competition with oxaloacetate binding at pH 8 may be consistent with the cMDH possessing a higher affinity for malate as the apparent K_i for the cMDH is 0.16 mM while the apparent K_i for the mMDH is 0.60 mM.

In addition to some differences in kinetic parameters for malate and oxaloacetate utilization by the two MDHs, some differences in NAD and NADH utilization are also observed (Fig. 2 and Fig. 4). At pH 9.2, apparent K_m's for NAD of 38 μM and 151 μM are observed for the c and mMDH from ribbed mussel gill tissue, respectively. The value for the cMDH is similar to the apparent Km value (10 μM) observed for the Patella caerulea cMDH (Lazou et al., 1987). NAD binding constants of MDHs form other sources include: 0.54 mM (K_m) for beef heart mitochondrial MDH (Grimm and Doherty, 1961) ; 0.75 mM (dissociation constant) for the chicken heart enzyme (Raval and Wolfe, 1962 a and b); and 0.48 mM (dissociation constant) for the mMDH and 0.59 mM (dissociation constant) for the cMDH from porcine heart (Holbrook and Wolfe, 1972). Differences in NAD utilization by MDHs from different cell compartments have been described previously. For the Opuntia MDHs, NAD apparent K_m's at pH 8.5 are 0.80, 0.79, and 1.9 mM for the chloroplastic, mitochondrial, and soluble forms, respectively. For spinach, apparent K_m's for NAD at pH 8.5 are 0.364, 0.574, and 0.232 mM for the microbody, mitochondrial, and soluble forms, respectively (Rocha and Ting, 1971).

The apparent K_m values observed for the c and mMDH for NADH are low at pH 8 (6.1μM and 4.5 μM for the c and mMDH, respectively) and increase

slightly when the pH is elevated to 9.2 (17μM and 6.6 μM for the c and mMDH, respectively). These values are comparable to binding constants for NADH observed for MDHs from diverse sources including: 25 μM (apparent Km) for the beef heart mMDH (Grimm and Doherty, 1961); 5 μM (dissociation constant) for the chicken heart enzyme (Raval and Wolfe, 1962 a and b); 2.16 μM (dissociation constant) for the cytosolic form and 1.4 μM (dissociation constant) for the mitochondrial form from porcine heart (Raval and Wolfe, 1962 a and b). At pH 7.4, apparent K_m's for NADH from the cactus are 62, 35, and 61 μM for the chloroplastic, mitochondrial, and soluble forms, respectively (Mukerji and Ting, 1969). For spinach, apparent K_m's for NADH of 24, 48, and 16 μM are observed for the microbody, mitochondrial, and soluble forms, respectively (Rocha and Ting, 1971). The apparent K_m for NADH for the Artemia nauplii is 15 μM (Hand et al., 1981). The cMDH from M. edulis mantle and adductor binds NADH in a cooperative manner (with the $S_{0.5}$ at 32 μM and Hill coefficient of 1.5) and concentrations of this substrate above 200 μM are inhibitory (Livingstone, 1976). NADH is similarly reported to be inhibitory to the beef heart mMDH also above 200 μM (Bracht and deCampillo, 1979). We find no evidence for substrate inhibition or cooperativity for the mMDH. Although some substrate inhibition and cooperativity may be present for the cMDH (Fig. 2), the data fit the hyperbolic kinetics model of Cleland as assessed by the low standard errors.

For both the cytosolic and the mitochondrial forms, bell shaped pH curves were obtained for oxaloacetate utilization, probably indicating the existence of two or more titrated amino acid residues at the active site (Fig. 5). The observed pH optima for the forward and the reverse directions are similar to

the values reported for the cytosolic enzyme from the foot of the gastropod P. caerulea (Lazou et al., 1987); the pH optima for malate and OAA utilization are 9.2 and 8.4, respectively for this cMDH. The pH optimum for oxaloacetate reduction is pH 8 for the cMDH from M. edulis (Livingstone, 1976). For oxaloacetate reduction, pH optima of 7.5 and 8.5 are observed for the c and mMDHs of the ribbed mussel, whereas pH optima of 9.5-10 are observed for both forms for malate oxidation.

Consistent with the mMDH possessing a slightly higher pH optimum for oxaloacetate reduction is the greater reversibility of this enzyme observed at pH 9.2 than is observed for the cMDH. Specifically, for the mMDH, the maximum rate of oxaloacetate reduction is about 5 times greater than the maximum rate of malate oxidation at pH 9.2, whereas for the cMDH, the maximum rate of oxaloacetate reduction is about 10 times greater than the maximum rate of malate oxidation at pH 9.2 (Table 1).

The higher pH buffer (pH 9.2) produces reliable results in measuring malate oxidation by MDHs (Wong and Smith, 1976). However, inclusion of 10 mM $MgCl_2$ as suggested by Wong and Smith raises the apparent K_m's for each of the various substrates. Part of this effect may be due simply to the increased ionic strength caused by the addition of this compound. Increased ionic strength has been observed to influence MDH activity from many species. It has several effects on the mitochondrial malate dehydrogenase from beef heart, including decreasing V_{max}, and increasing K_m values for NADH and OAA, and slightly increasing the K_i for NADH (Bracht and deCampello, 1979). While the beef heart mMDH exhibits a V_{max} effect, little

V_{max} effect is observed for the MDH from the oyster when the strength of the phosphate assay buffer is increased; however, increased phosphate buffer strength does have a competitive effect by increasing the K_m's for oxaloacetate and NADH (Sarkissian and Gomolinski, 1976). As with the mMDH and cMDH from the gill tissue of the ribbed mussel, mammalian mMDH appears to be more sensitive to inhibition by chloride derivatives than the cMDH. The basis of this greater sensitivity is the greater sensitivity of the mMDH to inhibition by the enol form of OAA (Bernstein et al., 1978). Chloride ions and other compounds including phosphate ions catalyze the tautomerization of oxaloacetate to the enol form (Gruber et al., 1956). The K_i's for chloride ions causing the inhibtion of oxaloacetate utilization were in the 30 mM range for the cMDH and the 6 mM range for the mMDH.

Different values for the apparent K_i's for $MgCl_2$ were determined for each of the various substrates at pH 9.2 with the DEA buffer system. The K_i's determined for oxaloacetate were lowest, possibly due to the effect of the chloride ion on the catalysis of the tautomerization of oxaloacetate (Gruber et al., 1956). The effect of $MgCl_2$ on the m and cMDHs is better studied with malate as substrate, as malate does not undergo the keto-enol tautomerization. Because $MgCl_2$ and Mg acetate are competitive inhibitors of malate utilization by the mMDH while sodium acetate apparently is a poor inhibitor (apparent K_i's of 8.05 and 11.24 mM), Mg^{2+} itself apparently is acting as the inhibitory species. Because the sodium derivatives of both chloride and acetate are inhibitors of the cMDH, as are $MgCl_2$ and Mg acetate, the role of Mg^{2+} on regulation of this enzyme is more difficult to understand. However, the apparent K_i's for $MgCl_2$ and Mg acetate (8.82 mM and 16.07 mM

respectively) are slightly lower than values of two times the apparent K_i's for sodium acetate and sodium chloride (29 and 36 mM). A Mg^{2+} effect may therefore be indicated. Regardless of the method of analysis (least squares vs. Cleland's method) and the range of concentrations of malate chosen, the $K_{i's}$ for Mg^{2+} inhibiton of malate by the c and mMDH are consistently determined to be between 8 and 16 mM. Because glutamate acts as a competitive inhibitor of malate at pH 9.2 and addition of $MgCl_2$ is found to enhance the glutamate effect, it is possible that both of these inhibitors operate at the dicarboxylic acid binding site. Consistently, the apparent K_i's for $MgCl_2$ inhibition of NAD utilization are larger than the values observed for malate binding (65.14 and 22.35 mM for c and mMDH, respectively) and the apparent K_i's for $MgCl_2$ inhibition of NADH utilization are larger than the values observed for oxaloacetate binding (9.42 mM and 34.10 mM for c and mMDH, respectively).

While the apparent K_i values for $MgCl_2$ inhibition of malate utilization are consistently calculated to be between 8 and 16 mM, the physiological relevance of this observation is uncertain. Marine invertebrates have higher concentrations of inorganic ions but the total inorganic ion composition of invertebrates is comparable with vertebrates (Pierce, 1982). Most Mg^{2+} within molluscan cells is bound (Burton, 1983). A typical value for the concentration of free Mg^{2+} is 2-3 mM (Baker and Crawford, 1972). However, the ionic composition of the bathing medium affects the ionic concentration of the body fluids of marine molluscs. Increases in Mg^{2+} in addition to Ca^{2+} and Na^{+} are observed when the ionic concentration of the medium increases (Shumway, 1977b). The increased ionic concentration of hemolymph may then cause an increase in the intracellular ionic composistion in tisssues

(Sarkissian and Gomolinski, 1976). Therefore, some regulation of MDH activity by Mg^{2+} and possibly Cl^- may occur in the ribbed mussel in response to changing environmental conditions.

The inhibition of the cMDH and mMDH from ribbed mussel gill by various organic acids (Figs. 6 and 7) indicates that these MDHs are similar to other MDHs. Aspartate, glutamate, succinate, fumarate, and pyruvate inhibit the MDH from plants when present in concentrations greater than 10 mM (Ting, et al., 1975). In the cactus (Opuntia sp.), MDHs from the chloroplastic, mitochondrial and cytosolic compartments are similarly inhibited by a broad range of dicarboxylic acids including alpha- ketoglutarate, cis-aconitate, citrate, isocitrate, succinate, fumarate, maleate, asparagine, glutamate (Mukerji and Ting, 1969). In contrast to the results for malate dehydrogenases from plant sources, the cMDH from the gastropod mollusc Patella caerulea is inhibited by alpha-ketoglutarate and citrate but not aspartate, fumarate, succinate, pyruvate, and fructose-1,6-bisphosphate (Lazou et al., 1987). The concentration of free amino acids in the cells of marine invertebrates can be 700-800 mM (Pierce, 1982; Costa et al., 1980). Therefore, inhibition by asparatate suggests a potential role of fluctuating levels of this potential metabolic intermediate in the regulation of cytosolic and mitochondrial MDHs. The mitochondrial forms's insensivity to alanine suggests that the production of high alanine levels during hyperosmotic and anaerobic stress (Paynter et al., 1984a) would not interfere with the continual functioning of the mMDH. Acetate production in the mitochondria during anaerobic stress (Ho and Zubkoff, 1983; deZwaan et al., 1981) also would not interfere with the functioning of this enzyme.

Hydroxymalonate is a competitive inhibitor of OAA utilization by the mMDH and is a non-competitive inhibitor of the cMDH (Fig. 8). Because hydroxymalonate acts competitively toward oxaloacetate and is uncompetitive toward malate with the pig heart mMDH, Harada and Wolfe (1968) suggest the existence of dead end enzyme-NADH-hydroxymalonate complexes. The K_i's for hydroxymalonate for the cMDH (1.17 mM) and the mMDH (0.256 mM) are much greater than the K_i for the mitochondrial malic enzyme (16 μM; see Chapter 4). These estimated values for hydroxymalonate binding to the mMDH are in good agreement with the results obtained for the porcine heart c and mMDHs. Holbrook and Wolfe (1972) report that the hydroxymalonate dissociation constant is highly dependent on pH, with the dissociation constant increasing from 45 to 620 μM for the mitochondrial form, as the pH is increased from 6.3 to 9.3 and the dissociation constant of the cytosolic form increasing from 520 to 4500 μM as the pH is increased from 6.3 to 9.3.

The difference between the apparent K_m's for NAD at pH 9.2 (38 μM for cMDH and 150 μM for mMDH; Fig. 4) and the apparent K_i's for NAD observed at pH 8 (0.60 mM for the cMDH and 0.82 mM for the mMDH; Fig. 9) may be partly attributed to a pH effect. However, a similar result is observed at constant pH by Heyde and Ainsworth (1968) who report a Ki_{slope} for NAD of 2.34 mM, whereas the apparent K_m is 60 μM. Consistently, Bernstein et al. (1978) report that a high concentration of NAD (10 mM) is necessary to cause a 50% inhibition of oxaloacetate reduction. That the apparent K_m's observed for NAD for MDHs from various sources, including the ribbed mussel

mMDH, are much less than the observed $K_{i's}$ may suggest that there is some regulation of NAD binding.

MDHs from both the cytosolic and mitochondrial compartments are regulated by adenine nucleotides (Lazou et al., 1987; Oza and Shore, 1973). While ATP is uncompetitive for oxaloacetate binding, ATP, ADP, and AMP at 2 mM are competitive inhibitors of NADH binding to the pig heart mMDH, (Oza and Shore, 1973). Under conditions of saturating NADH concentrations but low oxaloacetete, all three adenine nucleotides at 2 mM inhibited the P. caerulea cMDH by about 50% (Lazou et al., 1973). ATP also regulates the c and mMDH from the ribbed mussel. ATP is a competitive inhibitor of NADH binding to the cMDH with an apparent K_i of 0.77 mM. This result suggests that the nucleotide binding site of this enzyme is readily acccesible to ATP. Under conditions in which ATP concentration is decreased, as is observed during aerial exposure (Ebberink and deZwaan, 1980), this cMDH may become active at synthesizing malate for mitochondrial transport. ATP is a mixed non-competitive inhibitor of NADH binding to the mMDH. This may suggest some regulation of both ATP and NADH binding to this enzyme. From the 1 mM to 6 mM concentrations, the apparent K_i is estimated at 5.38 mM, indicating that this enzyme is relatively insensitive to ATP inhibition.

In addition to the c and mMDH from the ribbed mussel gill tissue's immunological distinction, the present study indicates that these forms are different kinetically. Specifically, the mMDH has a slightly higher affinity for OAA and also is more sensitive to substrate inhibition by OAA than the cMDH. The cMDH is more sensitive to substrate inhibition by malate than

the mMDH. The mMDH has a slightly higher pH optimum for OAA reduction than the cMDH, while the pH optima for malate oxidation by these enzymes are similar. The mMDH is more sensitive to inhibition by chloride ions than the cMDH. The cMDH is inhibited by alanine and sodium acetate. The cMDH behaves differently from the mMDH when inhibited with ATP. Also the mMDH is more sensitive to inhibition by hydroxymalonate than the cMDH. While the kinetic constants for OAA and NADH for the mMDH and cMDH are similar, the higher K_m's for malate and NAD exhibited by the mMDH may suggest that malate formed in the cytosol and transported into the mitchondria is not oxidized to OAA, but instead may be substrate for pyruvate synthesis by the malic enzyme or for anaerobic succinate synthesis by the fumarase/fumarate reductase reactions.

CHAPTER 3.

MALATE DEHYDROGENASE ISOZYMES FROM THE GILL TISSUE OF THE RIBBED MUSSEL

Abstract: Malate dehydrogenase from the gill tissue of ribbed mussels from the Cape Cod area is a polymorphic enzyme with two electrophoretically resolvable forms in the mitochondria (mMDH) and five resolvable forms in the cytoplasm (cMDH). The forms were separated by DEAE cellulose chromatography and identified by electrophoretic analysis. At pH 8, the apparent K_m's for oxaloacetate of each of the resolved cytosolic forms were between 28 and 90 μM. At pH 8, the resolved mitochondrial forms had apparent K_m's for oxaloacetate of 18 and 13 μM. Increasing the pH between the range of 6 to 8 had little effect on the apparent K_m's for all of the forms isolated from the cytosol with the exception of the most anodally migrating form. Increasing the pH over this range for both of the forms isolated from the mitochondria and the most anodally migrating form increased the apparent K_m for OAA. The apparent K_m's for OAA for all of the forms were greatly increased by elevating the pH of the assay mixtures to pH 9. The apparent K_m's for OAA for all forms could be increased by elevating the salt (NaCl) concentration in the assay mixtures from 0 to 100 mM to 200 mM; however, increasing salt raised the apparent K_m's for OAA of the mitochondrial forms to a greater extent than the apparent K_m's of most of the cytosolic forms. Both cMDH and mMDH are inhibited by high concentrations of OAA with the mMDH being more sensitive than the cMDH.

INTRODUCTION

Malate dehydrogenase (MDH) activity is found as distinct cytosolic and mitochondrial enzymes in most eukaryotic organisms studied (for review see Banaszak and Bradshaw, 1975), including several invertebrates (Drosophila sp.: McReynolds and Kitto, 1970; locust: Delbrück et al., 1959; shrimp: Hodnett et al., 1976; marine snail [Ilyanassa obsoleta]: Miezel and Markert, 1967; and sea urchin [Strongylocentrotus purpuratus]: Ozaki and Whitely, 1970). Plants have a third microbody-associated enzyme (Ting et al., 1975).

The uniqueness of the mitochondrial and cytosolic malate dehydrogenases from many sources is indicated by differences in peptide maps from these two enzymes (Kitto and Kaplan, 1966), slight differences in amino acid composition (Siegel and Englard, 1962; Kitto and Kaplan, 1966), immunological distinction (Kitto and Lewis, 1967; Kitto, 1967; McReynolds and Kitto, 1970), and some kinetic differences. Kinetic differences include the ability of only the mitochondrial form to use several 2-hydroxydicarboxylic acids other than malate (Davies and Kun, 1957; Englard and Brieger, 1962), substrate inhibition of cMDH by malate (Kun and Volfin, 1966; Delbrück et al., 1959; Abou-Zamzam and Wallace, 1970; Englard and Breiger, 1962; Siegel and Englard, 1961; Kitto and Kaplan, 1966), and the greater sensitivity of the mMDH to substrate inhibition by oxaloacetate (Kitto and Kaplan, 1966; McReynolds and Kitto, 1970; Siegel and Englard, 1961; Englard and Breiger, 1962).

Malate dehydrogenases associated with the cytosolic and mitochondrial compartments have distinct electrophoretic mobilities on native gels (Thorne et al., 1963; Ting et al., 1975). Therefore, electrophoresis of total cell proteins from most organisms which possess unique cMDH and mMDH usually produces multiple MDH bands. While two bands are expected on native gels of entire cell proteins (one for cMDH and one for mMDH), often many more bands are observed.

The presence of multiple bands resolved on native gels from cytosolic preparations may be at least in part explained by either the presence of a single genetic locus with at least two alleles or by the presence of more than one genetic locus (Davidson and Cortner, 1967b; Bailey et al., 1969). However, when more than one locus with multiple alleles is present, the interpretation of cMDH patterns becomes complex (see for example peach plants [Arulsekar et al., 1986]; snails [Biomphalaria glabrata: Narang and Narang, 1974]; Ascaris sp. [Zee et al., 1970]).

For the several marine molluscs studied to date, the number of genetic loci reported ranges between one and five (Fujio et al., 1983; Ayala et al., 1973; Buroker, 1983; Koehn and Mitton, 1972; Koehn et al., 1988). One locus with two alleles for the cMDH is observed for both Modiolus demissus and Mytilus edulis (Koehn and Mitton, 1972) making these among the simpler molluscan MDH systems. For dimeric proteins encoded by a nuclear locus, such as cMDHs from a number of sources, the presence of two alleles in an interbreeding population results in the appearance of three distinct banding patterns (or phenotypes) on native gels of homogenates derived from single

individuals: slow/slow homozygotes, fast/fast homozygotes, and heterozygotes with both the fast/fast and slow/slow forms plus a third electrophoretically distinct form, the fast/slow heterodimer (Hubby and Lewontin, 1966; Karig and Wilson, 1971; Davidson and Cortner, 1967b; Bailey et al., 1969). In both M. edulis and M. demissus, their respective fast alleles are observed to predominate; therefore the phenotype observed on native gels for the majority of individuals of both species is fast/fast. While heterozygotes are observed, both species demonstrated a conspicuous absence of individuals which were homozygous for the slow allele (Koehn and Mitton, 1972). Because the cMDH pattern does not vary in response to latitude and therefore with ambient temperature in M. edulis (Koehn et al., 1976), and does not vary in response to salinity in M. edulis and M. demissus (Koehn and Mitton, 1972), there may be little evolutionary pressure on the cMDH locus. These results are in contrast to results obtained for the cMDH pattern of the killifish which varies with latitude (Powers and Place, 1978; Cashon, 1982) and an LAP locus of bivalves which varies with salinity (Koehn and Mitton, 1972).

The presence of the fast/fast, slow/slow, and fast/slow forms of cMDH from the pooled cytosol of several M. demissus individuals should produce three bands on native gels. However, the observed result is a far more complex pattern (Paynter et al., 1985a) and the basis for this complex pattern is not understood. That at least part of cMDHs observed complex pattern is due to the interchangeability of some conformational forms is suggested by a study on MDH from another mollusc (Ilyanassa obsoleta), in which treatment with mercaptoethanol caused seven resolvable forms to coalesce into a single

band (Meizel and Markert, 1967). In further support of the interconvertibility of electrophoretically resolvable forms, Demenéch et al. (1987) observed that MDH isozymes found in purified cMDH from several guinea pig and chicken tissues, when resolved to homogeneity by isoelectric focusing, would revert to the original complex mixtures with aging.

Polymorphism has also been reported for mitochondrial malate dehydrogenase for many diverse organims (including barley cotyledons [Grimmwood and McDaniel, 1970]; pig heart [Thorne et al., 1963]; several bird species [Kitto et al., 1966a]; rat liver [Kuan et al., 1987]; rabbit heart [Dölken et al., 1974]; and humans [Davidson and Cortner, 1967a]). The various mMDHs resolvable by native gel electrophoresis differ little in catalytic properties, possess similar resistance to thermal inactivation (Kitto et al., 1966b) and possess very similar amino acid composition (Kitto et al., 1966a). While mitochondrial electromorphs exhibit similar physical properties, some striking differences are also observed including: differential sensitivity to iodine and hydroxymercuribenzoate, different optical rotational properties, and differential ability to recover from reversible denaturation with guanidine-HCl (Kitto et al., 1966a; Schecter et al., 1968). These differences may be consistent with one or several mMDH molecular species existing in several different conformational states (Kitto et al., 1966a, 1966b). However, polymorphism in mMDH also appears to have a genetic basis in human white blood cells (Davidson and Cortner, 1967a). In sequencing of mMDH from rat heart, Grant et al. (1987) observed that one amino acid residue could not be unambiguously identified. Because treatment of resolved mMDH electromorphs with guandine-HCl did not affect electrophoretic mobility, it is

not likely that the existence of the several mMDH bands is simply due to the alteration in confomational state of a single molecular form (Schechter et al., 1968).

In bivalve molluscs, the cMDH and mMDH are important in the regulation of carbon flux between the cytosol and mitochondria for organic acid (succinate and propionate) biosynthesis during anaerobiosis (deZwaan et al. 1981), for cellular osmolyte (alanine) biosynthesis during hyperosmotic stress (Baginski and Pierce, 1977; Bishop et al., 1981) and for gluconeogenesis (deZwaan et al, 1983a). The mMDHs have a central role in the tricarboxylic acid cycle (deZwaan et al., 1983a). Kinetic information on MDHs from molluscs, especially mMDHs, is limited and their similarities to MDHs from other sources is not well documented (see for oyster MDH: Sarkissian and and Gomolinski, 1976; M. edulis MDH: Livingstone, 1976; P. caerulea MDH: Lazou et al., 1987). Studies with MDHs from other sources indicate that they are susceptible to regulation by pH (Zschoche and Ting, 1973) and ionic strength (Kun et al., 1967; Sarkissian and Gomolinski, 1976; Place and Beynon, 1982). The altered concentration of intracellular metabolites that accumulate under environmental stress may also be important in the regulation of malate dehydrogenase (Kun and Volfin, 1966; Delbrück et al., 1959; Abou-Zamzam and Wallace, 1970; Siegel and Englard, 1962). Because of the complex nature of the cMDH and mMDH patterns from the ribbed mussel observed on starch gels and the uncertain basis of their polymorphisms, the kinetics of each of the electrophoretically distinct forms needs to be evaluated.

The following reports the separation of the electrophoretically distinct forms of the cMDHs and mMDHs from the ribbed mussel by DEAE cellulose chromatography and evaluates the reactivity of these forms with OAA under regimes of increasing pH and salt (NaCl) in the assay mixtures.

MATERIALS AND METHODS

Animals: Ribbed mussels (Modiolus demissus) were purchased from Northeast Environmental Laboratories (Monument Beach, Mass) and were maintained in artificial seawater (Jungle Laboratories, Inc., Sanford, Fla.) as described previously (Greenwalt and Bishop, 1980).

Reagents: Substrates, buffers, chromatographic and electrophoretic supplies, and reagents were purchased from Sigma Chem. Co. (St. Louis, MO.). Monobasic sodium phosphate and sodium chloride were purchased from Fisher. Ammonium sulfate (ultra pure, special enzyme grade), and sucrose (special enzyme grade) were purchased from Schwarz/Mann. Boric acid purchased from Matheson, Coleman, and Bell. Miracloth was obtained from Calbiochem.

Identification of isozymes from crude cytosol and mitochondrial preparation and fractions homogeneous for these isozymes: Isozyme profiles from the cell compartments were examined by starch gel electrophoresis. The electrode buffer for this gel contained Tris (15.1 gm/l) boric acid (1.5 gm/l) and EDTA (1.5 gm/l) adjusted to pH 7.0 with glacial acetic acid (see Harris & Hopkinson's 1976 recipe for the aspartate aminotransferase system). The gel buffer used was a 1:l0 dilution of running buffer and 10% (w/v) starch was used. Crude cytosol and crude sonicated mitochondria were dialyzed in 5 mM $NaP0_4$ pH 6.5 then applied to sample wells and electrophoresed at 35 mAmps for approximately 6.5 hours until Bromphenol blue marker in outer lanes reached the end of the gel. Gels were sliced horizontally and stained

with 50 ml solution containing 50 mM L-malic acid in 0.1M Tris pH 8.0. To the staining solution, 10 mg NAD, 7.5 MTT, and 5 mg PMS were added. Staining was allowed to proceed overnight at 23°C.

Enzyme Assay: During purification and standard assay, MDH activity was determined spectrophotometrically by measuring the oxidation of NADH to NAD^+ at 340 nm at room temperature (23°C). The reaction mixture for the standard assay consisted of 20 µl enzyme added to 2.00 ml of a solution containing 50 mM HEPES, 3.03 mM OAA, and 258 µM NADH prepared just prior to use. Reaction rates are expressed as $\Delta O.D._{340}$/minute under these conditions.

Kinetic analysis of OAA utilization was determined over a variety of pH's (6,7,8,9) in a solution containing 50 mM HEPES of the stated pH, 180 µM, NADH, varying concentrations of an aqueous OAA solution (used within three hours of preparation) and 20-50 microliters of enzyme solution for a final volume of 2.00 ml. In addition to the effects of pH on OAA utilization, the effect of increasing NaCl concentration at pH 7.0 was also investigated.

Kinetic constants for OAA utilization were determined using Cleland's (1979) substrate inhibition program, while constants for OAA utilization in the presence of 100 and 200 mM NaCl at pH 7.0 were determined using Hill plots with least squares analysis.

Enzyme preparation: All procedures were performed at 0-4°C. Gills were removed from mussels and collected in a chilled, tarred beaker until approximately 35 gm of tissue had been accumulated. This tissue was rinsed

in cold sea water and homogenized in 10 ml/gm tissue mitochondrial isolation buffer (0.5 M sucrose, 20 mM K-HEPES, 1 mM K-EGTA, pH 7.5 with KOH) with an "Ultra-Turrax" homogenizer (Tekmar, Cincinatti, Ohio) using 3-10 sec bursts on "40" setting. The homogenate was filtered through 1 layer of Miracloth, with Miracloth replaced after approximately 200 milliliters. The filtrate was centrifuged at 1500 x g for 10 minutes and the pellet was discarded. The supernatant was recentrifuged at 9,000 x g for 15 minutes to pellet the mitochondria. The resulting supernatant was frozen at -20°C until ready for use in purification of cytosolic isozymes. The pellet from this step was demonstrated by Burcham et al. (1984) to contain mitochondria. The mitochondria contained within this pellet were rinsed by resuspension in homogenization buffer, and repelleted by centrifugation at 9,000 x g for 15 minutes. This second pellet was resuspended in 100 mM $NaP0_4$ pH 8.0 then frozen at -20°C until ready for use in purification of the mitochondrial isozymes.

Aliquots of the mitochondria were thawed and sonicated with 2-30 second blasts at setting "5" of a Branson sonicator. The sonicated preparation was centrifuged at 15,000 x g for 20 minutes, and the pellet resuspended, resonicated, centrifuged and the supernatants combined.

Aliquots of thawed cytosol (100 ml) and sonicated mitochondria (total mitochondrial preparation) were taken through a similar purification procedure. Both preparations were first brought to 30% saturation with the slow addition of powdered solid $(NH_4)_2S0_4$ at 4°C, stirred for 2 hours at 4°C, the precipitate was removed by centrifugation at 15,000 x g for 20 minutes.

This supernatant was brought to 70% $(NH_4)_2SO_4$ saturation, stirred for 2 hours at 4°C then centrifuged as above and the pellet was resuspended in 5 mM $NaPO_4$ pH 6.5. This preparation was dialyzed against 2 changes of deionized water of approximately 20 times volume of resuspended pellet followed by 20 volumes of 5 mM $NaPO_4$ (pH 6.5). The dialyzed preparation was chromatographed on a DEAE cellulose column equilibrated in the same buffer measuring 1.5 x 25 cm. After loading, the column was washed with the same buffer until the A_{280} of collected fractions was near baseline, at which time a linear 0 - 250 mM NaCl gradient (400 ml) made up in the same buffer was applied to elute the various isozymes of MDH. Activity of the eluted fractions was determined using the standard assay procedure.

To resolve coeluting cytosolic isozymes which had the greatest anodal migration on native gels, active fractions were pooled, dialyzed, and rechromatographed on the DEAE cellulose column by washing with a solution containing 100 mM NaCl in 5 mM $NaPO_4$ pH 6.5, then applying a second linear 100-400 mM NaCl gradient (400 ml) in 5 mM $NaPO_4$, pH 6.5. Similarly, less mobile coeluting isozymes could be resolved by pooling, dialyzing, and rechromatographing on the DEAE cellulose column using a linear 0 - 100 mM NaCl gradient (400 ml) gradient in this same buffer.

Fractions identified as being homogenous for a particular isozyme were pooled and dialyzed against 20 x volume 5 mM $NaPO_4$, pH 6.5 overnight and stored at 4°C. Preparations were stable for several days under these conditions.

RESULTS

DEAE Cellulose Chromatography and Starch Gel Electrophoresis: Starch gel electrophoresis of crude cytosol from pooled gill tissue revealed five isozyme forms, whereas mitochondrial preparations revealed two (Fig. 1). All of these forms appear to be electrophoretically unique. Resolution of the isozymes from cytosol (denoted C1 through C5 in order of increasing anodal mobility) and mitochondria (denoted M1 and M2 with M2 having the greater anodally mobility) was achieved by DEAE cellulose chromatography and examples of typical chromatograms are presented in Figures 2 a,b & c and Figure 3. As there was a substantial amount of coincident elution of adjacent isozymes observed in most fractions collected from the columns, yields of electrophoretically homogeneous preparations were typically low but enough enzyme of each form was obtained for the kinetic analyses. A representative gel showing the separation of the various forms is included (Fig. 4).

Kinetic Analyses of the Separated "Electromorphs": The low apparent Km's for OAA observed at pH 8 for each of the five forms observed in the cytosol (28-90 μM) and the two forms observed in the mitochondria (17 and 13 μM) are presented in Table 1. The C1 form had the lowest apparent K_m for OAA of all of the cytosolic MDHs under these conditions. There appeared to be no statistically significant difference between forms C3 and C4. The apparent K_m of the C2 form was intermediate between those observed for the C1 and C3-C4 forms. The apparent K_m of the C5 forms was lower than the apparent Km of C4. There was little difference between the apparent K_m's for OAA of the two MDH forms resolved from the mitochondria (M1 and M2).

Figure 1. Native Starch Gel Pattern of MDH Forms from the Cytosol and Mitochondria of the Gill Tissue of the Ribbed Mussel. See Materials and Methods for conditions.

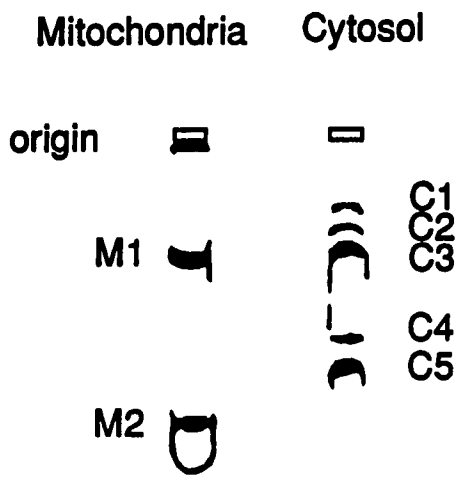

Mitochondria
Cytosol
origin
M1
M2
C1
C2
C3
C4
C5

Figure 2. Chromatographic Separation of Cytosolic MDH Isozymes

Figure 2a. Resolution of the C1 Form. Each fraction contained approximately 4 ml.

Figure 2b. Resolution of C1, C2, and C3. Each fraction contained approximately 2 ml.

Figure 2c. Resolution of C4 and C5. Each fraction contained approximately 2 ml.

Conditions of operation are reported in Materials and Methods.

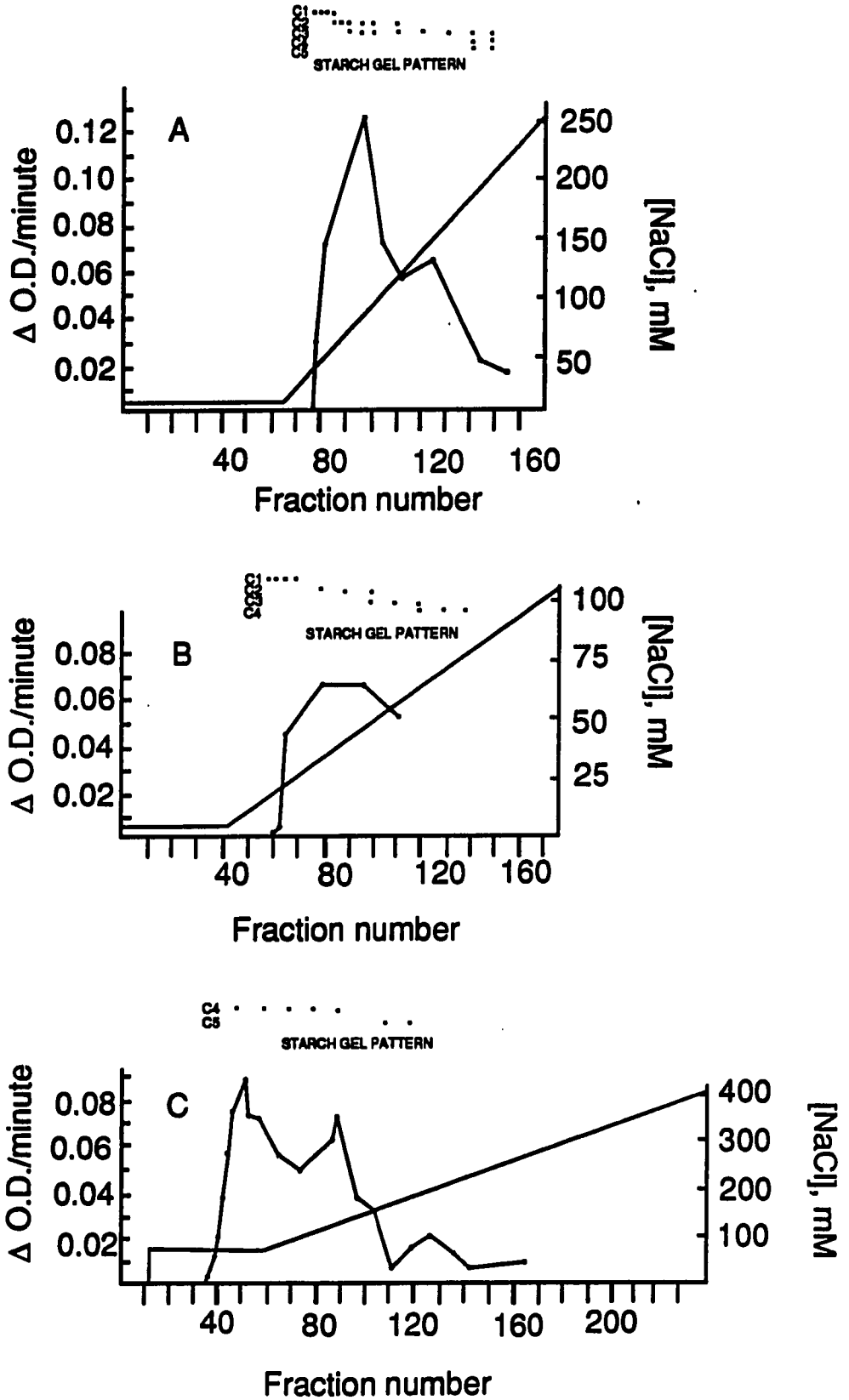

STARCH GEL PATTERN
A
Δ O.D./minute
0.12
0.10
0.08
0.06
0.04
0.02
250
200
150
100
50
[NaCl], mM
40
80
120
160
Fraction number
STARCH GEL PATTERN
B
Δ O.D./minute
0.08
0.06
0.04
0.02
100
75
50
25
[NaCl], mM
40
80
120
160
Fraction number
C4
C5
STARCH GEL PATTERN
C
Δ O.D./minute
0.08
0.06
0.04
0.02
400
300
200
100
[NaCl], mM
40
80
120
160
200
Fraction number

Figure 3. Chromatographic Resolution of the Mitochondrial Malate Dehydrogenase Isozymes. Each fraction contained approximately 3 ml. Conditions of operation are given in Materials and Methods.

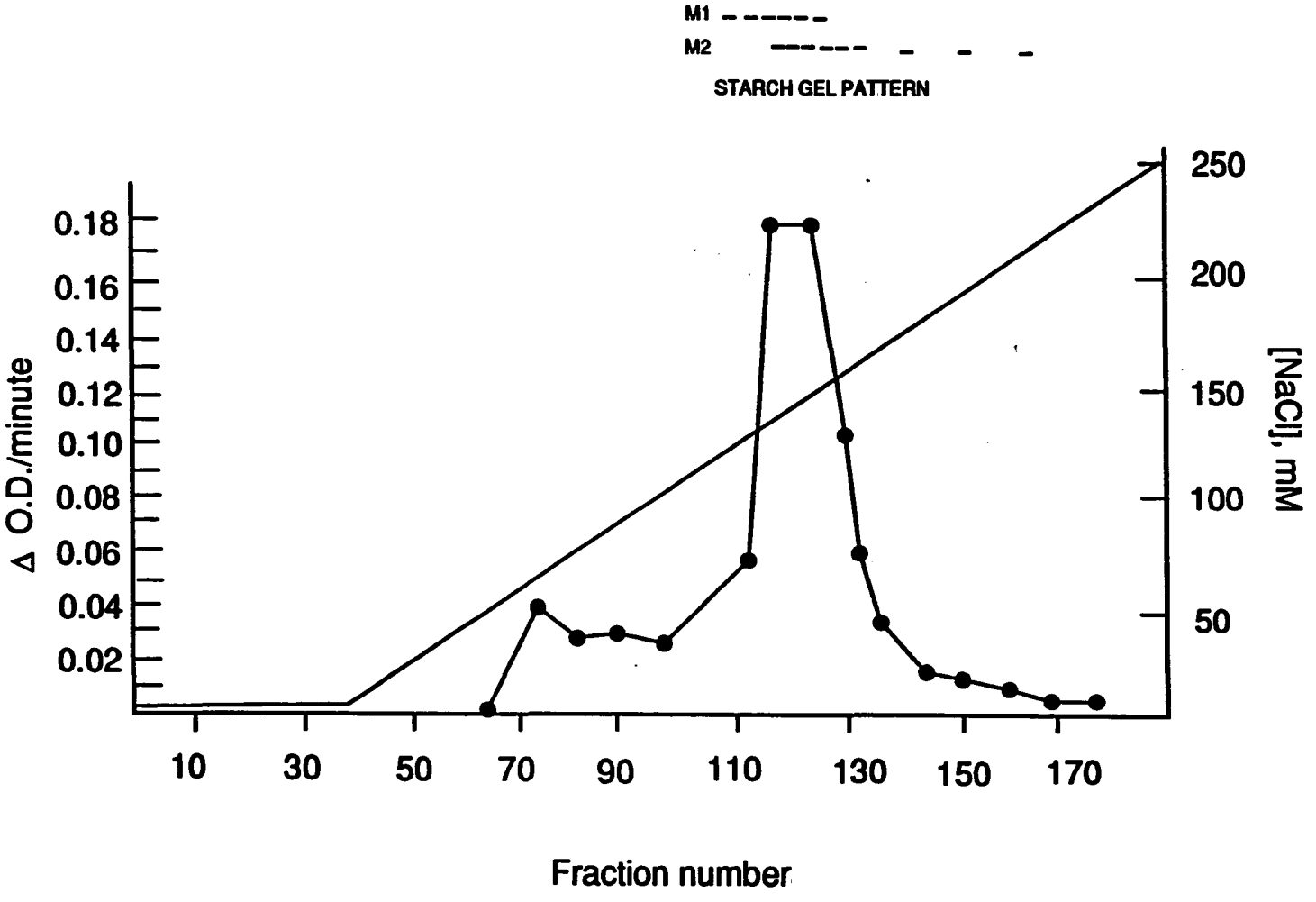

M1 ------
M2 ------ - - -
STARCH GEL PATTERN
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
Δ O.D./minute
250
200
150
100
50
[NaCl], mM
10
30
50
70
90
110
130
150
170
Fraction number

Figure 4. Resolution of cMDH by DEAE Cellulose Revealed by Starch Gel Electrophoresis.

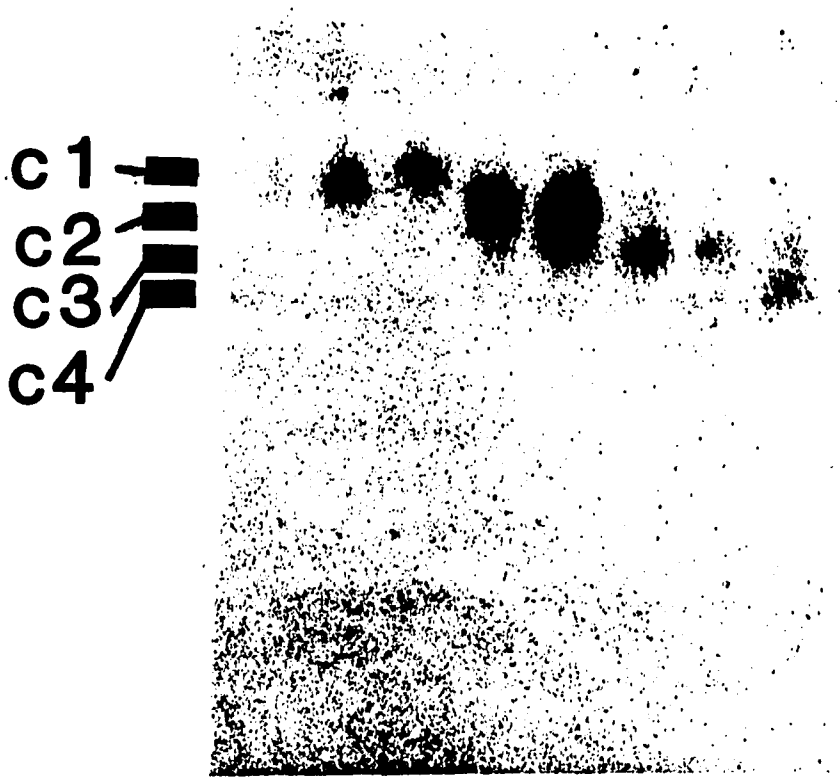
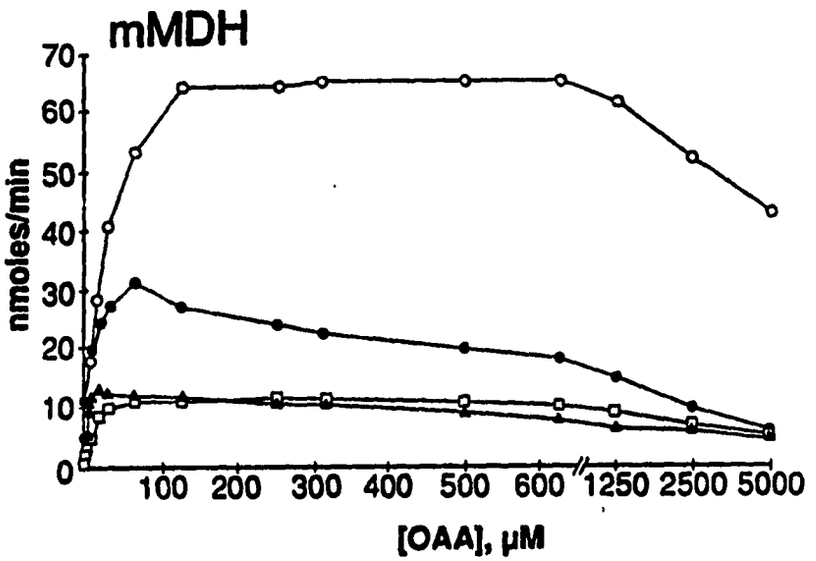

c1
c2
c3
c4

Table 1. Summary of the Apparent K_m's for MDH Forms from the Ribbed Mussel Gill Tissue[a]

Form	Apparent K_m (μM) ±standard error
C1	28.32±2.89
C2	53.55±6.03
C3	84.25±14.26
C4	89.77±15.94
C5	46.00±4.80
M1	17.44±4.64
M2	12.77±1.68

[a]Reaction mixtures contained enzyme, 50 mM HEPES (pH 8), 180 μM NADH and variable concentrations of OAA ranging from 1 to 5,000 μM.

Figure 5. Effect of pH on Oxaloacetate Reactivity of the Cytosolic and Mitochondrial MDH's from the Ribbed Mussel. Partially purified C1 and partially purified M2 were used as the cMDH and mMDH, respectively. Reaction mixtures contained enzyme, 50 mM HEPES buffer of the indicated pH, and constant NADH (215 μM) in a final reaction volume of 2.00 ml. (Closed triangles: pH 6. Open squares: pH 7. Closed circles: pH 8. Open circles: pH 9.)


mMDH
70
60
50
40
30
20
10
0
nmoles/min
100 200 300 400 500 600 1250 2500 5000
[OAA], µM

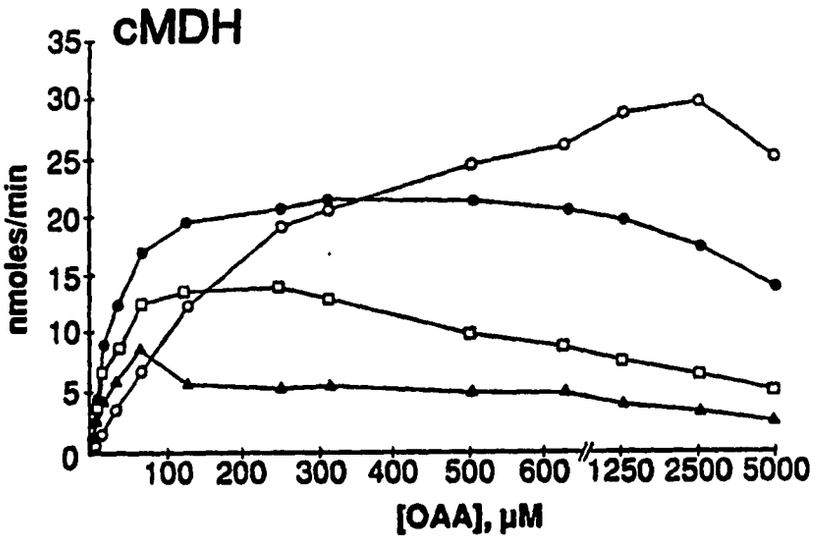

cMDH
35
30
25
20
15
10
5
0
nmoles/min
100 200 300 400 500 600 1250 2500 5000
[OAA], µM

Table 2. The Effect of pH on the Observed K_i (Substrate Inhibition) Value for Oxaloacetate for the C1 and M2 MDH Isozymes

	Apparent K_i (mM)[a]			
Isozyme Form	pH 6	pH 7	pH 8	pH 9
C1	0.97±0.40	1.26±0.40	6.30±0.94	10.03±1.20
M2	0.81±0.15	0.71±0.10	1.42±0.22	5.92±0.44

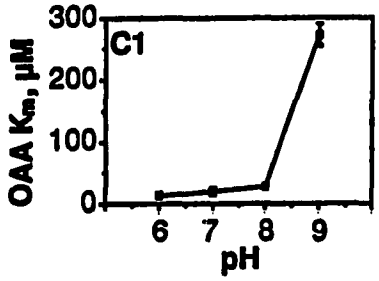
[a]Values were calculated with Cleland's substrate inhibition computer program from data shown in Figure 5.

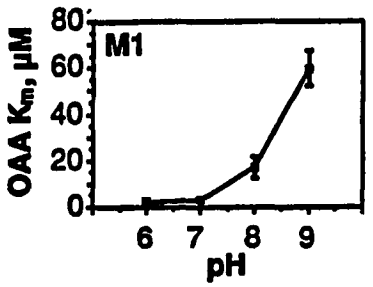
The apparent K_m's of these two forms were lower than the apparent K_m's of any of the cytosolic forms.

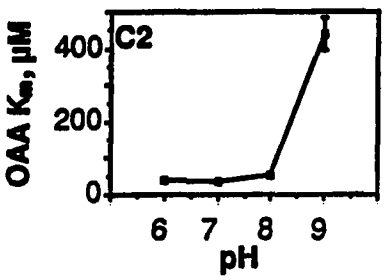
Increasing the pH of the assay mixture for both the C1 and M2 isozymes affected OAA binding (Fig. 5). The calculated K_i's for the mitochondrial isozyme M2 and the cytosolic isozyme C1 from the data shown in Figure 5 indicate that the mitochondrial isozyme is much more sensitive to OAA substrate inhibition than is the cytosolic isozyme (Table 2). Similar patterns of increasing pH affecting OAA binding are observed for each of the resolved forms with the general effect of increasing the K_m for OAA for each of the MDH forms (Fig. 6). The data summarized in Figure 6 indicated that forms C1 through C4 showed little change in affinity for OAA as the pH's of the assay mixtures were increased from 6 to 8 as assessed by the calculated apparent K_m values. Forms C5, M1, and M2 were similar in that increasing the pH of the assay mixtures from 6 to 8 increased the apparent Km's for OAA. The K_m's for OAA for all forms showed sharp increases when the pH was increased from 8 to 9.

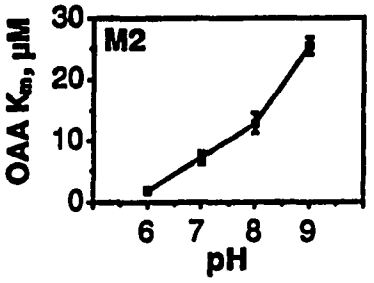
To further discriminate between MDH forms, the variations in the apparent K_m's for OAA at pH 7 with increasing NaCl concentrations were evaluated. Increasing NaCl concentration increased the Km for OAA for all forms, and caused non-linear slightly parabolic 1/velocity vs. 1/substrate plots at the highest concentrations of NaCl (200 mM) used in these studies (Fig. 7). This result was most pronounced at higher substrate concentrations (ie lower 1/[OAA] values). Because of the non-linearity of these plots, Hill plots were used to estimate $K_{0.5}$ values. Increased NaCl raised the $K_{0.5}$ for all forms

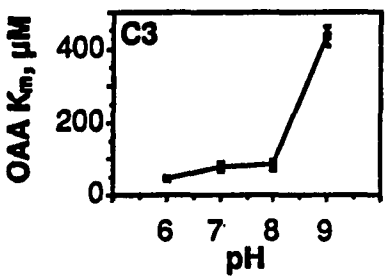
Figure 6. Effect of Increasing pH on the Oxaloacetate Utilization. Reaction mixtures contained enzyme, 50 mM HEPES at the indicated pH, 180 μM NADH, and various quantities of OAA ranging from 1 to 500 μM.

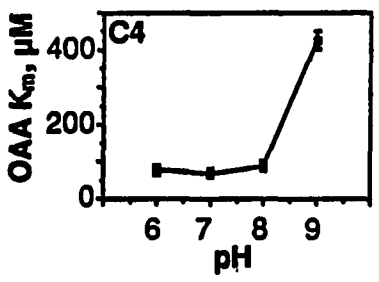

C1
OAA Km, µM
300
200
100
0
6 7 8 9
pH


M1
OAA Km, µM
80
60
40
20
0
6 7 8 9
pH


C2
OAA Km, µM
400
200
0
6 7 8 9
pH


M2
OAA Km, µM
30
20
10
0
6 7 8 9
pH


C3
OAA Km, µM
400
200
0
6 7 8 9
pH


C4
OAA Km, µM
400
200
0
6 7 8 9
pH

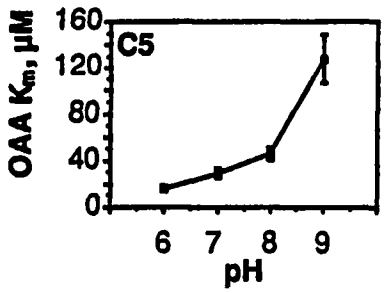
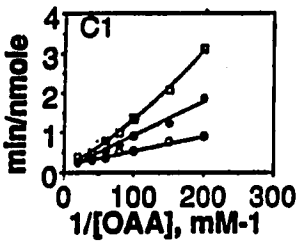
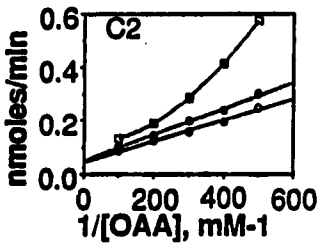
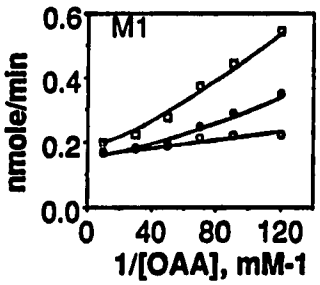
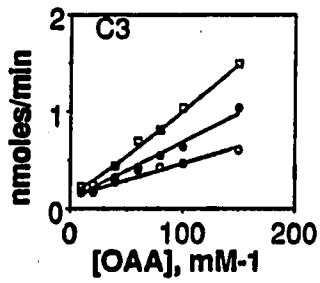

C5
OAA Km, µM
160
120
80
40
0
6 7 8 9
pH

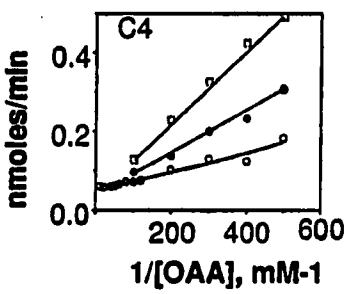
Figure 7. Effect of Increasing NaCl Concentration on Each Malate Dehydrogenase Form from Ribbed Mussel Gill Tissue. Reaction mixtures contained enzyme, 50 mM HEPES (pH 7), 180 μM NADH, and the indicated concentrations of OAA. Open circles: assays contained 0 NaCl; Closed circles: assays contained 100 mM NaCl; Open squares: assays contained 200 mM NaCl.

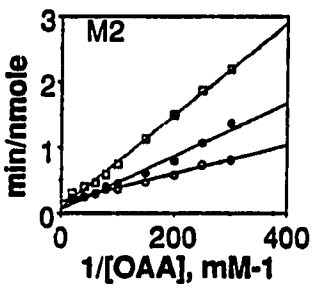

C1
min/nmole
0
1
2
3
4
0
100
200
300
1/[OAA], mM-1


C2
nmoles/min
0.0
0.2
0.4
0.6
0
200
400
600
1/[OAA], mM-1


M1
nmole/min
0.0
0.2
0.4
0.6
0
40
80
120
1/[OAA], mM-1


C3
nmoles/min
0
1
2
0
100
200
[OAA], mM-1


C4
nmoles/min
0.0
0.2
0.4
200
400
600
1/[OAA], mM-1


M2
min/nmole
0
1
2
3
0
200
400
1/[OAA], mM-1

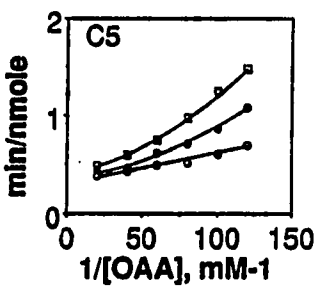

C5
min/nmole
0
1
2
0
50
100
150
1/[OAA], mM-1

Table 3. Effect of Increasing NaCl Concentration on the apparent K_m for OAA[a]

	Apparent K_m(μM)		
Form/[NaCl]	0 NaCl[b]	100 mM NaCl[c]	200 mM NaCl[c]
C1	22.84	26.04	57.35
C2	36.04	77.43	85.54
C3	78.42	94.45	119.79
C4	55.51	113.47	164.87
C5	17.75	21.87	32.56
M1	2.29	9.05	18.17
M2	6.83	9.98	23.39

[a]Values calculated from data in shown in Figure 6. Reaction rates were measured at pH 7 in these experiments.
bData were calculated using Cleland's (1979) computer program.
cBecause of the non-linear replots of 1/V vs 1/[S], Hill plots were used to calculate these values.

(Table 3). As judged by the extent of the increase in the $K_{0.5}$, the mitochondrial forms appeared to be more sensitive to inhibiton by NaCl than the cytosolic forms.

DISCUSSION

The complexity of malate dehydrogenases resolved on native gels from a number of sources has prevented a thorough understanding of this enzyme system. In this study with ribbed mussel gill tissue, five distinct forms are found in the cytosol and two are found in the mitochondria (Fig. 1). The various forms are resolvable by DEAE cellulose chromatography (Figs 2, 3, and 4). The apparent K_m values observed for the ribbed mussel gill MDHs are comparable to apparent K_m values of MDHs from other sources (Table 1 and Fig. 6). Some representative K_m values for malate dehydrogenases from a variety of sources include 74 and 125 μM for the pea epicotyl cytosolic and mitochondrial forms, respectively (Davies, 1969), 50 μM and 38 μM for the chicken heart cytosolic and mitochondrial MDH, respectively (Kitto and Kaplan, 1966), 34 μM for the beef heart mitochondrial enzyme (Siegel and Englard, 1962), 50 μM for the E. coli enzyme (Murphy et al., 1967) and 160 μM for the B. subtilis enzyme (Murphey et al., 1967).

While little difference in the apparent K_m's are observed for MDHs from the cytosol and mitochondria of most organisms, the ribbed mussel is unique in that the apparent K_m's for OAA of the mitochondrial forms are much smaller than the apparent Km's of the cytosolic forms (Table 1). In addition, differences between the various MDH forms isolated from the cytosol are apparent (Fig. 6, Fig. 7 and Tables 1 and 3). A previous report on MDH polymorphism in M. demissus (Koehn and Mitton, 1972) found that the fast/fast cytosolic form is most prevalent in the population studied, while there were very few individuals which were homozygous for the slow/slow

allele. As our gels were run at the same pH, it is likely that the form that we identify as C1 corresponds to the cytosolic form found in slow/slow homozygous individuals and C3 or C4 corresponds to the cytosolic form found in fast/fast homozygous individuals. The form we designate as C2 may represent the heterodimeric form found in heterozygous individuals along with C1 and C3 or C4. The differences in the apparent K_m's between C1, C2, C3-C4 and C5 indicate that these various forms are genuinely different and are not simply interchangable forms. While differences are found among the cytosolic enzymes, little difference was observed between the kinetic properties of the two mMDHs from the ribbed mussel.

Cytosolic MDH has been purified and characterized from several invertebrate sources including Artemia sp. (Hand et al., 1981), and Patella caerulea (Lazou et al., 1987); however, the Artemia sp. and possibly the P. caerulea preparation have several MDH forms with different mobilities on native gels (Bowen and Sterling, 1978; Hand et al., 1981). The apparent K_m value obtained for the Artemia enzyme at pH 8 (42 μM) and the P. caerulea enzyme at pH 8 (36 μM) may represent averages of the K_m's of the several forms present.

Increasing pH of the assay mixture from 6 to 9 increases the apparent K_m's for all forms examined; however most of the cytosolic forms (C1 through C4) are relatively insensitive to regulation by pH over the range the physiological range compared to the mitochondrial forms (Fig. 6). This effect of increasing pH on increasing the apparent K_m for the enzyme has been observed for several other MDHs (Zshoche and Ting, 1973). The cMDH from P. caerulea

appears to be more sensitive to regulation to pH over the physiological range than the C1 through C4 cMDHs from M. demissus (Lazou et al., 1987).

The effect of ionic strength in raising the $K_{0.5}$ for OAA (Table 3 and Fig. 7) has been shown for MDH from the oyster (Sarkissian and Gomolinski, 1976). That the mitochondrial form is more sensitive to inhibition by increased ionic strength is a typical characteristic of vertebrate MDHs (see Kun et al., 1967). Exactly how the ionic strength affects the activity of MDH is not known, but Place and Beynon (1982) report that changes in ionic strength causes conformational changes in the protein which are apparent as alterations in thermal lability and chromatographic properties. With some of the MDH isozymes (Fig. 7), the higher salt concentrations produce obviously non-linear Lineweaver-Burk plots which may be consistent with a conformational change of the enzymes. An alternative explanation is that this inhibition is due to the chloride ion catalyzed enolization of oxaloacetate (Gruber et al., 1956) which produces a recognized inhibitor of the enzyme (Bernstein et al., 1978). While NaCl was used to increase the ionic strength of the assay mixture in this study of the ribbed mussel gill MDHs, increased phosphate buffer concentration was found to have a similar effect on OAA reactivity as well as NADH reactivity in the study on oyster MDH (Sarkissian and Gomolinski, 1976). Phosphate ion also catalyzes the enolization of OAA (Gruber er al., 1956). Although the mechanism of the salt effect is uncertain, salt may be an extremely important regulator of this enzyme activity under hyperionic stress as suggested by Sarkissian and Gomolinski (1976).

The observed inhibition of a mitochondrial malate dehydrogenase by high concentrations of oxaloacetate (Table 2) may be due to the formation of abortive complexes between the enol form of oxaloacetate and the enzyme-NADH complex (Bernstein et al, 1978). Consistent with results reported previously for malate dehydrogenase from beef heart and other sources (Englard and Breiger, 1962; Davies and Kun, 1957; Delbrück et al., 1959), the ribbed mussel gill mMDH is more sensitive to substrate inhibition by high concentrations of OAA than is the cMDH form examined as indicated by the lower K_i of the mMDH for this substrate. In contrast to the results of Kun and Volfin (1966) who found only the mitochondrial MDH to undergo substrate inhibition, we found all forms of MDH from the ribbed mussel gill to be inhibited at OAA concentrations exceeding 250 μM. The lower K_i for OAA observed for the mitochondrial form of the enzyme may be consistent with the lower apparent K_m's observed for this form.

The lower K_m's of the mitochondrial enzymes suggest that in the mitochondrion, a small quantity of oxaloacetate accumulating there from any number of sources may drive the formation of malate. This might be especially important when the supply of acetyl CoA becomes limiting, as during shutdown of the pyruvate dehydrogenase reaction (Paynter et al., 1985b). Malic enzyme, which catalyzes the synthesis of pyruvate from malate has been observed to be primarily, if not exclusively, mitochondrial in the ribbed mussel (Paynter et al., 1985a). In conjunction, alanine aminotransferase appears to be exclusively mitochondrial (Paynter et al., 1984a). These results suggest a possible pathway for formation of the alanine

observed to accumulate in the mitochondria during aerobic osmotic stress via the formation of malate from oxaloacetate or the transport of malate across the mitochondrial membrane, malate's decarboxylation to form pyruvate, and finally pyruvate's transamination to form alanine.

CHAPTER 4.

MITOCHONDRIAL MALIC ENZYME FROM RIBBED MUSSEL GILL TISSUE: SEPARATION FROM MALATE DEHYDROGENASE, NATIVE MOLECULAR WEIGHT, REACTIVITY WITH METAL IONS, AND OTHER PROPERTIES

Abstract: Mitochondrial malic enzyme (ME) was partially purified by ammonium sulfate precipitation of sonicated ribbed mussel gill tissue mitochondria and gel filtration on Sephadex G-150. The mME had an apparent M_r = 265,000 and limited ability to use NAD as a cofactor (~2% of the rate with NADP). The pH optima for malate and pyruvate utilization were pH 8.5 and 6, respectively. The apparent K_m's for malate were low at pH's 6 and 7 and high at the optimal pH's. A divalent metal ion was required for activity; with EDTA-divalent metal ion buffers at pH 7, $S_{0.5}$ values for metal ion-ME complexes were 18 picomolar, 2 picomolar, and 140 μM with Mn^{2+}, Co^{2+}, and Mg^{2+}, respectively. Increasing the concentration of Mn^{2+} decreased the apparent K_m for malate and the cooperativity associated with malate reactivity. Alkali chloride salts and sodium acetate were inhibitory with apparent K_i's in the 20-50 mM range. Increasing the concentration of malate decreased the apparent K_m for NADP. Pyruvate was a competitive inhibitor of malate utilization (K_i=4.5 mM). NADPH was a competitive inhibitor of NADP utilization (K_i= 16 μM). The malate analogue hydroxymalonate was a mixed inhibitor of malate oxidation and a non-competitive inhibitor of pyruvate reduction (apparent K_i's = 16 μM and 147 μM, respectively). Succinate (5 mM) was only slightly inhibitory, whereas fumarate (5 mM) was a more potent inhibitor. ATP (up to 3 mM) was found to have no effect on malate binding. when assayed with saturating (10 mM) $MnCl_2$. Acetyl, propionyl, and butyryl CoA at 100 μM caused 20-25 % inhibition of the malic enzyme reaction.

INTRODUCTION

The widespread distribution of malic enzyme (L-malate: NADP oxidoreductase, E.C.1.1.1.40), or ME, has been noted (Frenkel, 1975); however, this activity has only been studied in partially purified preparations from a few molluscan species, including oyster (Crassostrea virginica) adductor muscle (Hochachka and Mustafa, 1973), sea mussel (Mytilus edulis) mantle and adductor muscle (deZwaan and van Marrewijk, 1973) and squid mantle muscle (Storey et al., 1975). The intracellular distribution of ME, a feature which is linked to its function, has been determined for a few invertebrate species. The ME activity in crustacean, helminth and annelid tissue is mitochondrial (Skorkowski et al., 1977; Li et al., 1972; Landsperger et al, 1978; Hoffmann et al., 1979). ME activity is localized to the cytosol of the oyster adductor muscle (Hochachka and Mustafa, 1973), the sea mussel hepatopancreas, and the sea mussel mantle, but is about equally distributed between the cytosolic and mitochondrial compartments of the sea mussel adductor muscle (deZwaan and van Marrewijk, 1973). ME is found localized to the mitochondria of the gill tissue from several species including the ribbed mussel, oysters, and quahogs, but is localized to both the cytosolic and the mitochondrial compartments of the gill tissue of the blue mussel (Paynter et al., 1985a).

The ME from the ribbed mussel has a relatively low (0.02 U/mg mitochondrial protein) specific activity (Paynter et al., 1985a). This specific activity is considerably smaller than values reported for parasitic helminthes which use ME-derived reduced nucleotides to drive the mitochondrial

soluble compartment colocalized fumarate reductase to produce ATP (Hymenolepis sp.: Fioravanti, 1982; McKelvey and Fioravanti, 1985; Ascaris suum: Fodge et al., 1972). However, the ribbed mussel has in common with the parasitic helminthes the production of succinate and other volatile fatty acids in response to limiting oxygen availability (Ho and Zubkoff, 1983). Specifically, succinate is synthesized rapidly in response to anaerobic stress possibly by an activity colocalized with malic enzyme similar to the mitochondrial fumarate reductase described in the closely related blue mussel (Holwerda and deZwaan, 1980). Malic enzyme in the ribbed mussel may have a role in the production of reduced nucleotides for succinate production. Longer exposure causes the accumulation of large concentrations of propionate and smaller quanitities of lactate, acetate, butyrate, and isobutyrate (Ho and Zubkoff, 1982, 1983). The processes leading to the production of some of these other volatile fatty acids in response to anaerobic stress have been best described in parasitic helminthes (Tkachuck et al., 1977; vanVugt et al., 1979; Komuniecki et al., 1979). In molluscs, the production of succinate, propionate, and acetate is linked to the metabolism of malate and asparate carbon skeletons (deZwaan et al., 1981; Collicut and Hochachka, 1977). The importance of malate as a precursor to these compounds is also indicated by the observation that malate serves as an excellent respiratory fuel for isolated mitochondria, whereas pyruvate either is not transported or else does not stimulate O_2 consumption in a coupled manner (Burcham et al., 1984; Ballantyne and Moon, 1985). Mitochondrial pyruvate metabolism is indicated by the formation of accumulating acetate, implicating the active

pyruvate dehydrogenase located in this cell compartment (van Vugt et al., 1979; Paynter et al., 1985b).

While several volatile fatty acids accumulate during anaerobic stress, alanine is observed to accumulate under both conditions of anaerobic stress (Ho and Zubkoff, 1982) and hyperosmotic stress (Baginski and Pierce, 1977, 1978). Several lines of evidence indicate that alanine is derived in molluscs from the four carbon precursor aspartate. Specifically, in the ribbed musssel adapted to 12‰, alanine rises from 20 micromoles to 140 micromoles/gram dry weight after 6 hours of exposure to 32‰ concomitant with aspartate levels dropping from 40 micromoles to 20 micromoles/gram dry weight (Baginski and Pierce, 1977), and labeled asparate supplied to isolated oyster ventricles under anaerobic conditions accumulates in alanine (Collicut and Hochachka, 1977). That malate may serve as an intermediate in the formation of alanine from aspartate is suggested by the lack of an active LDH in the tissues of many molluscs which must then rely on the cytosolic malate dehydrogenase reaction (using the aspartate transamination product oxaloacetate as an electron acceptor) to compensate for alterations in redox balance (Hochachka et al., 1973; Fields, 1983) which occur during both anaerobiosis and hyperomotic stress (Baginski and Pierce, 1975). Direct evidence for malate serving as a precursor for alanine is the distribution of carbon from malate supplied to isolated anaerobic mitochondria from the sea mussel into alanine (deZwaan et al., 1981).

Alanine synthesis is mitochondrial as the only activity which can produce alanine <u>de novo</u> in molluscs, the alanine aminotransferase, is solely

mitochondrial in the ribbed mussel gill tissue (Paynter et al., 1984a). Because of the mitochondrial co-localization of the ME and the alanine aminotransferase (Paynter et al., 1985a), and the importance of malate as a respiratory fuel to the anaerobic mitochondrion, the kinetics of the malic enzyme are of interest in understanding the processes leading to alanine biosynthesis as well as the biosynthesis of volatile fatty acids.

As propionate, acetate, and other fatty acids are synthesized in parasitic helminthes from their CoA derivatives, and CoA dervivatives have been reported to regulate ME activity from other sources, the effect of these derivatives on the ribbed mussel gill mitochondrial ME is of interest. The potential regulatory roles of fumarate and succinate are also of interest, since these compounds are synthesized from malate by isolated anaerobic sea mussel mitochondria (deZwaan et al., 1981) and have also been reported previously to modulate the activity of MEs from several sources. The possible role of ATP regulation which has been described for other mitochondrial MEs is also considered.

The sensitivity of the ribbed mussel mitochondrial ME to the malate analogue hydroxymalonate is considered, as this inhibitor has a potential use in the in vivo inhibition of this malic enzyme. Use of this compound has demonstrated the role of the high activity ME of cod heart mitochondria in malate metabolism (Skorkowski et al., 1984). This compound may be useful in demonstrating that the ME from ribbed mussel gill mitochondrial is important in the synthesis of pyruvate for alanine synthesis and as a substrate for the PDH reaction (Paynter et al., 1985b). The PDH product, acetyl CoA, is a

likely precursor for the small amount of acetate that accumulates during anaerobic stress possibly by serving as a CoA donor in the synthesis of succinyl CoA from succinate (van Vugt et al.,1979).

MATERIALS AND METHODS

Animals: Ribbed mussels (Modiolus demissus) were purchased from Northeast Environmental Laboratories (Monument Beach, MA) and maintained as described by Greenwalt and Bishop (1980).

Chemicals and Reagents: All reagents were purchased from Sigma Chemical Co., St. Louis, MO., with the following exceptions: ammonium sulfate (enzyme grade) was purchased from Schwartz-Mann, $MnCl_2$, and $CoCl_2$ were purchased from J.T. Baker Chemical Co., and $MgCl_2$ was purchased from Fisher Scientific.

Enzyme Assay: One unit of activity is defined as that amount of enzyme capable of catalyzing the formation of 1 μmole of product per minute. During the purification, ME activity was monitored by measuring NADPH formation in a Beckman 3600 recording spectrophotometer at room temperature (23°C) in a reaction mixture containing 50 mM HEPES of the indicated pH (see Results), with 156 μM NADP, 10 mM sodium malate, 100 μM $MnCl_2$ and enzyme in 2.0 ml. The reverse reaction (malate formation) for pH optimum determination was measured by following NADPH oxidation in reaction mixtures containing 50 mM MES at the indicated pH (see Results) with 10 mM sodium pyruvate, 160 μM NADPH, 25 mM sodium bicarbonate, 100 μM $MnCl_2$ and enzyme in 2.0 ml. Nucleotide specificity was determined by replacing NADP in the reaction mixture with an equal concentration of NAD.

Malate dehydrogenase activity was determined by measuring NADH oxidation in a reaction mixture containing enzyme, 3.03 mM sodium oxaloacetate, 258 μM NADH, and 50 mM HEPES (pH 8).

For studies examining the reactivity with various cations, reaction mixtures contained 50 mM HEPES (pH 7), 10 mM sodium malate, 156 μM NADP and the indicated concentrations of EDTA and divalent cations in 2.0 ml (see Results). The concentrations of the free (non-EDTA bound) divalent metal ions in the EDTA metal ion buffer systems were estimated using the "Chelate" program (Sherwin Lee, University of Pennsylvania) which is based on the equations developed by Steinhardt et al. (1977) using the stability constants of Martell (1964). The stability constants used for K1 through K4 for H,L to HHH,L were 10.23, 6.16, 2.67, and 1.99. The K1 and K2 for Co,L and Co,HL were 16.21 and 3.09, respectively. The K1 and K2 for Mn,L and Mn,HL were 14.04 and 6.90, respectively. The K1 and K2 for Mg,L and Mg,HL were 8.69 and 2.28, respectively.

The contents of assay mixtures used for inhibitor studies are described in individual figure legends.

Protein concentrations were determined as described by Miller (1959) with bovine serum albumen as a standard.

Enzyme Preparation: All procedures were performed at 2-4° C. Gill tissue from thirty ribbed mussels was removed, weighed, then homogenized twice in 10 vol/weight of ice cold homogenization buffer (0.4 M sucrose, 20 mM K-HEPES, and 1 mM K-EGTA, pH 7.5) using an Ultra-Turax (Tekmar) at a power

setting of "45" for 10 seconds. The homogenate was filtered through one layer of Miracloth (Calbiochem) and the filtrate centrifuged at 9,000 x g for 15 minutes. The supernatant was decanted and centrifuged at 9,000 x g for 15 minutes. The supernatant from this centrifugation was denoted cytosol and frozen (-20°C). The pellet (shown to contain mitochondria: Burcham et al., 1984) was resuspended in 100 mM sodium phosphate (pH 6.8) and sonicated three times for 20 seconds each using a Branson Sonic Power sonicator on a setting of "8". Powdered ammonium sulfate was added to the sonicate to a concentration of 30% saturation with constant stirring. The mixture was centrifuged for 20 minutes at 15,000 x g, and the supernatant brought to 70% saturation by the addition of ammonium sulfate powder then stirred for one hour at 4°C. The mixture was centrifuged and the supernatant discarded. The resulting pellet was dissolved in 5 ml of 100 mM sodium phosphate (pH 6.8) and dialyzed overnight against 1 L of this same buffer. The dialyzed preparation was applied to a Sephadex G-150 column (4.5 x 36 cm) which had been equilibrated in the same buffer and calibrated with molecular weight standards [apoferritin, amylase, alcohol dehydrogenase, bovine serum albumen, and carbonic anhydrase (Sigma Chemical Co.)]. The fractions from the Sephadex column were analyzed for enzyme activity and protein content (Fig. 2). ME containing fractions which lacked OAA dependent NADH oxidation (MDH) activity were pooled for use in the kinetics experiments. Aliquots (200 µL) of this partially purified preparation contained within 100 mM sodium phosphate buffer were used in the enzyme assays.

Where $MnCl_2$ concentration was increased to 1 mM or greater, the phosphate buffer used throughout the purification procedure was removed

by dialysis of the enzyme preparation against a stabilizing storage buffer consisting of 10 mM Tris (pH 7.0), 0.5 mM DTT, and 20 % glycerol as indicated in figure legends.

Data Analysis: Enzyme Assays: Enzyme assays were performed in duplicate with generally less than 10 % difference between replicates. Assays were performed on a Beckman 3600 recording spectrophotometer at 23°C. For sigmoidal kinetics, Hill plots were prepared and the slopes ("n" values) and the $S_{0.5}$ values were obtained by least squares analysis. For the clearly hyperbolic NADP kinetics, the statistical program of Cleland (1979) was used. For the inhibitor studies, data were analyzed by least squares analysis to obtain K_m, slope, and K_i values (Segel, 1975).

Native Gel Electrophoresis: Gill tissue from several ribbed mussel individuals were prepared by method of Klier (1988) with a 30 second homogenization of 1 ml of buffer per 500 mg tissue (2:1 volume per weight). Each 100 ml of homogenization buffer contained 88 mg ascorbic acid, 600 mg dibasic sodium phosphate, 52 mg disodium ethylenediaminetetracetate, 1 ml ß-mercaptoethanol, 7.2 g sucrose, and 5.0 g PVP (40,000 MW average). The pH was adjusted to 7.5 with NaOH. The homogenate was centrifuged in a microfuge tubes in an Eppendorf microfuge for 5 minutes. Fifteen microliter aliquots were applied per lane to a 7% acrylamide gel with Tris (pH 8.8) as the gel buffer and Tris-glycine as the running buffer. Samples were electrophoresed for 6 hours at 15 mAmps, until bromophenol blue markers applied to outer lanes had reached the bottom of the gel. Half of the gel was stained for malic enzyme activity, as described in Harris and Hopkinson

(1976), and the other half was stained as described for malic enzyme with NAD replacing NADP as the nucleotide cofactor.

RESULTS

A considerable amount of ME activity was found in the cytosolic fraction of the gill tisue homogenates, probably indicating mitochondrial breakage. ME activity had previously been found to be mitochondrial in this tissue (Paynter et al., 1985a). The specific activity in the mitochondrial fraction (0.061 U/mg) was much greater than that in the cytosolic fraction (0.0066 U/mg) (Table 1).

The malic enzyme and cytosolic and mitochondrial malate dehydrogenases were identifiable by native gel electrophoresis (Fig. 1). Malic enzyme activity appeared to use only NADP as a cofactor. Several distinct malic enzyme mobilites were observed by native gel electrophoresis. Several of the individuals possessed two distinct malic enzyme bands. The cytosolic malate dehydrogenases appeared to be able to use both NAD and NADP as cofactors, whereas the mitochondrial malate dehydrogenase only used NAD as a cofactor. While most of the individuals assayed possessed on mitochondrial malate dehydrogenase band and one cytosolic malate dehydrogenase band, one of the individuals appeared to be heterozygous for cytosolic malate dehydrogenase.

The NADP-specific malate utilizing activity (ME) in the 30-70% saturated ammonium sulfate fraction of the mitochondrial sonicate that was applied to the Sephadex G-150 column eluted as a single peak (Fig. 2) with an apparent M_r = 265,000 (Fig. 2 inset). This purification produced a 14-fold increase in the specific activity of the ME (Table 1). As seen in the chromatogram (Fig. 2),

Table 1. Partial Purification of Malic Enzyme from the Ribbed Mussel[a]

Step	Volume (ml)	Unit/ml[b]	Total U	Protein[c] (mg/ml)	Total Protein (mg)	Specific Activity	Purification (fold)	Yield
Cytosol	310	0.018	5.5	2.8	860	0.0066	---	---
Mitochondrial Sonicate	100	0.038	3.8	0.62	61.5	0.061	1	100%
30-70% $(NH_4)_2SO_4$	10	0.29	2.9	1.46	14.6	0.20	3.2	77%
Sephadex	30	0.057	1.7	0.066	2.0	0.86	14	45%

[a]Gill tissue of 31 individuals used as starting material.
[b]One unit is defined as that quantity of enzyme catalyzing the formation of one μmole of product per minute under the conditions specified.
[c]Protein concentration was determined by method of Lowry.

Figure 1. Native Gel Electrophoresis of Malate Utililizing Activities from the Gill Tissue of the Ribbed Mussel.

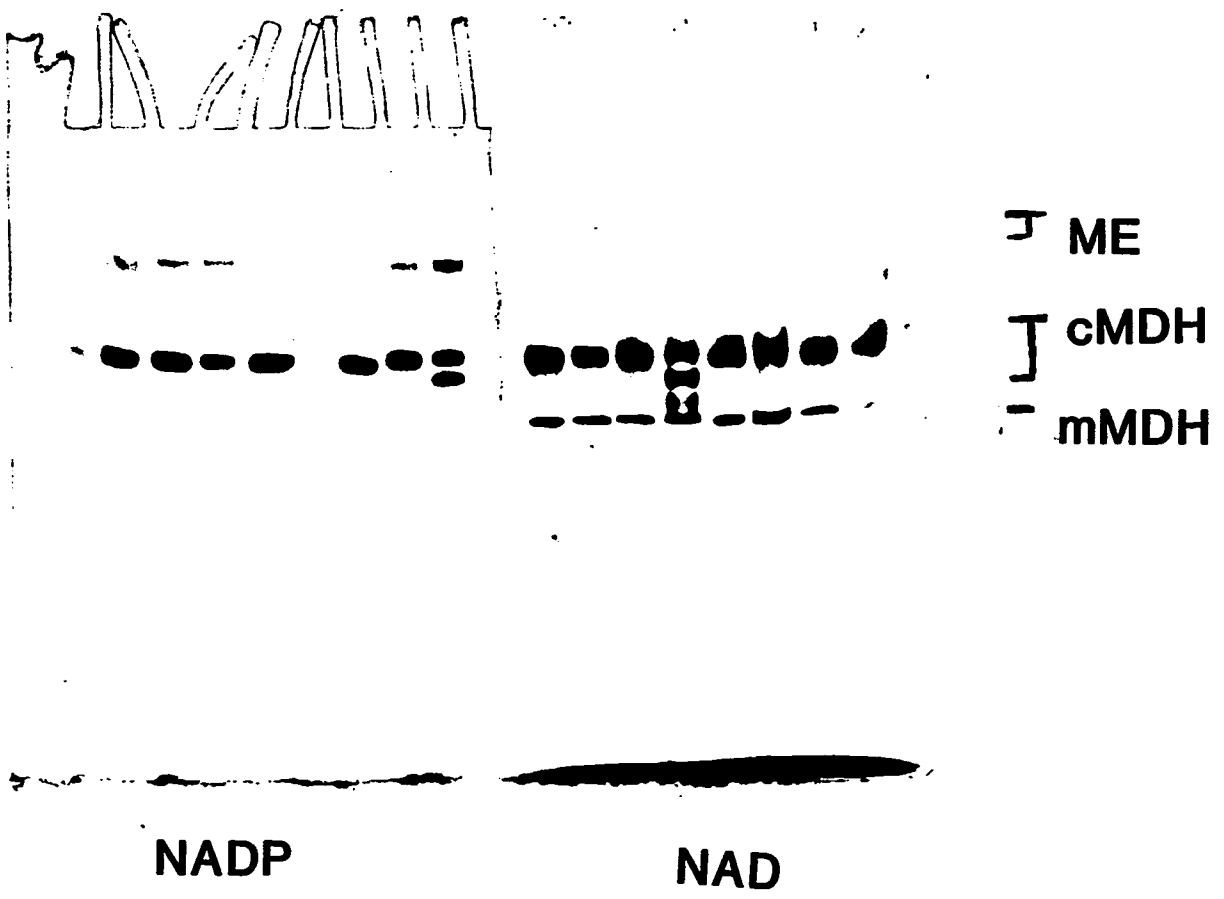
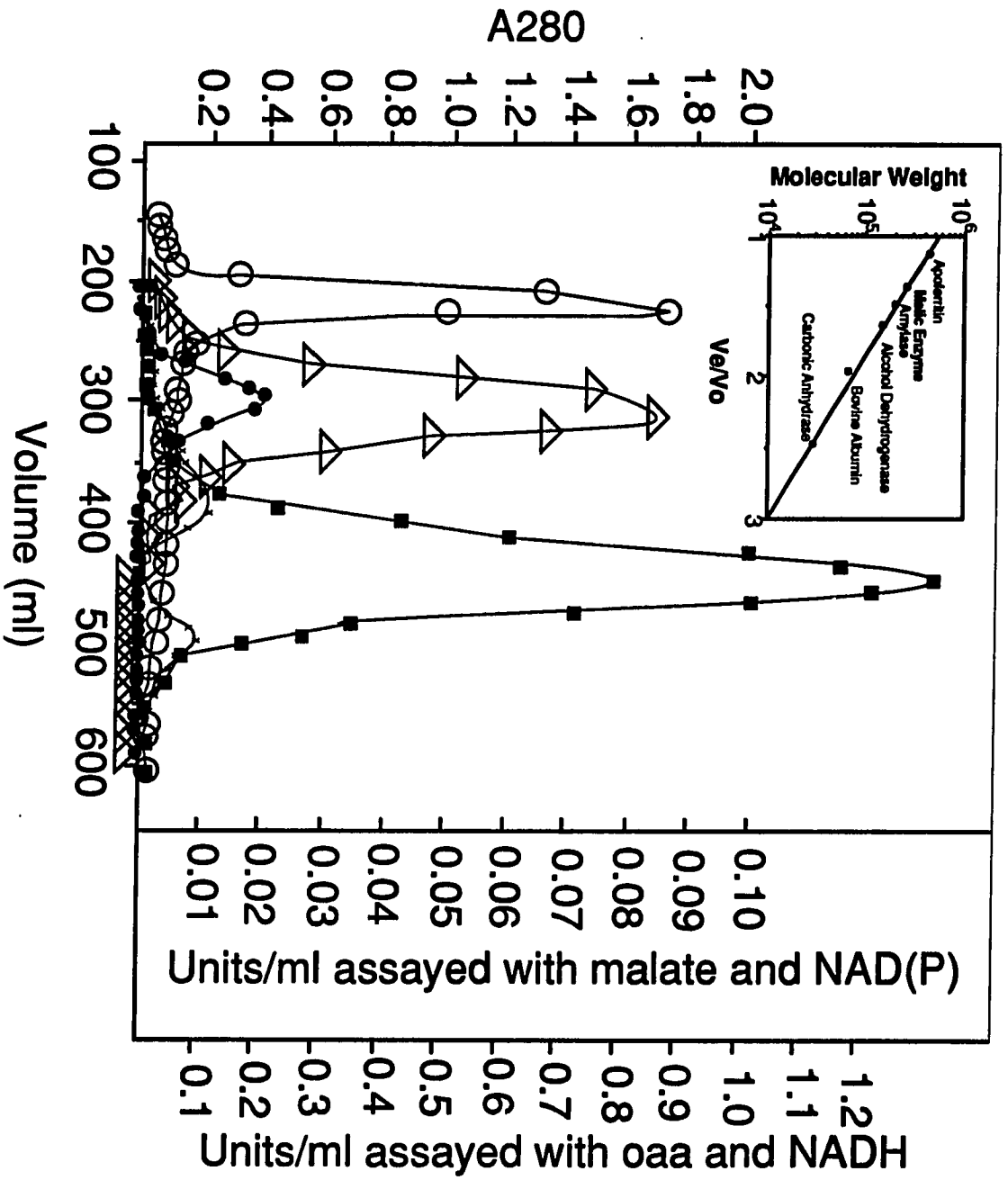

ME
cMDH
mMDH
NADP
NAD

Figure 2. Sephadex G-150 Elution Profile of Malate Utilizing Enzymes from the Mitochondria of Ribbed Mussel Gill Tissue. Ammonium sulfate precipitated mitochondrial proteins were resuspended in a small volume and applied to a Sephadex G-150 column (4.5 x 36 cm). Fractions were analyzed for absorbance at 280 nm (O - O). Malate dependent NADP reduction by column fractions was determined by addition of two hundred microliter aliquots to reaction mixtures containing 50 mM HEPES pH 8, 10 mM sodium malate, 156 μM NADP plus 100 μM $MnCl_2$ (Δ - Δ) or minus 100 μM $MnCl_2$ (● - ●). Malate dependent NAD reduction by column fractions was determined by addition of two hundred microliter aliquots to reaction mixtures containing 50 mM HEPES pH 8, 10 mM sodium malate, 156 μM NAD plus 100 μM $MnCl_2$ (X-X). Oxaloacetate dependent NADH oxidation by column fractions (determining the location of MDH elution) was measured by addition of 25 μl aliquots to reaction mixtures containing 50 mM HEPES (pH 8), 3.03 mM OAA, and 258 μM NADH (■-■).

Inset. Native Molecular Weight Determination of Gill Tissue mME by Sephadex G-150 Gel Filtration. The native molecular weight of the NADP utilizing peak was determined.


A280
Molecular Weight
Ve/Vo
Apoferritin
Malic Enzyme
Amylase
Alcohol Dehydrogenase
Bovine Albumin
Carbonic Anhydrase
Volume (ml)
Units/ml assayed with malate and NAD(P)
Units/ml assayed with oaa and NADH

fractions eluting in this molecular weight range contained an activity that would reduce NAD to NADH at about 2% of the rate of NADP reduction. Some malate dependent NADP-reduction was observed when $MnCl_2$ was omitted from the reaction mixtures (Fig. 2). The ME activity eluted from the column was stable for less than one week when stored in the 100 mM phosphate buffer at 4°C. After dialysis against 20 mM Tris (pH 7), 0.5 mM DTT, and 20% glycerol, the ME activity was stable for up to four months when frozen at -20°C.

Two small peaks of malate dependent NAD reducing activity (Fig. 2) eluted from the Sephadex G-150 column. The first peak of this malate dependent NAD reducing activity (V_o, 300-430 ml) had some NADP cross reactivity while the second did not (V_o, 450-560 ml). Enzyme in both peaks oxidized NADH in the presence of OAA indicating both peaks possessed some MDH activity. The OAA dependent NADH oxidizing activity eluted at V_o 400-500 ml with an apparent M_r = 60,000.

Preliminary characterization of the mME activity: Optima of pH 8.5 and 5-7 were observed for the forward (malate utilizing) and reverse (malate producing) reactions, repectively (Fig. 3), when assayed under fixed substrate concentration assay conditions. As the pH of the assay mixtures increased between 6 and 9, the V_{max} increased and the apparent K_m for malate increased (Fig. 4). With the low $MnCl_2$ concentration (100 μM) used in these assays, the rate with increased malate concentration increased in a sigmoidal fashion at all pH's. Hill coefficients for the four curves were 3.55, 1.97, 1.77, and 2.06 at pH's 6, 7, 8, and 9, respectively. As the pH increased, the apparent $S_{0.5}$ for

Figure 3. Determination of pH Optimum for the Ribbed Mussel Gill mME. Reaction rates in the malate utilizing direction (NADPH forming) were measured with reaction mixtures containing enzyme, 50 mM HEPES of the indicated pH's, 10 mM sodium malate, 156 μM NADP, and 100 μM $MnCl_2$ (open symbols). Reaction rates in the pyruvate utilizing direction (NADP forming) were measured with reaction mixtures containing enzyme, 50 mM MES of the indicated pH's, 10 mM sodium pyruvate, 160 μM NADPH, 25 mM sodium bicarbonate, and 100 μM $MnCl_2$ (closed symbols).

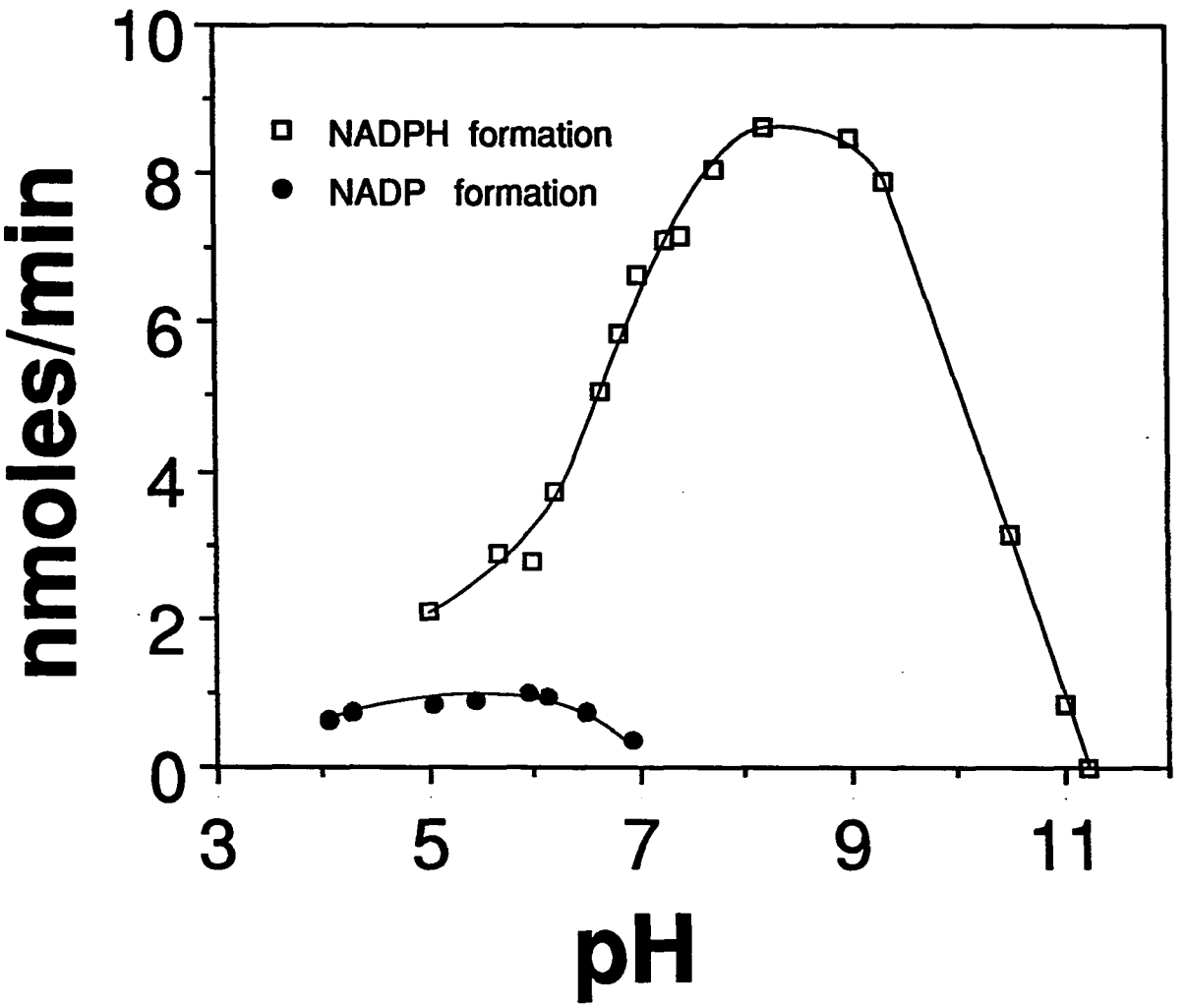

nmoles/min
10
8
6
4
2
0
NADPH formation
NADP formation
3
5
7
9
11
pH

Figure 4. The Effect of pH on Malate Utilization. Reaction mixtures contained enzyme, 50 mM HEPES of the indicated pH's, 156 μM NADP, 100 μM $MnCl_2$, and the indicated concentrations of sodium malate.

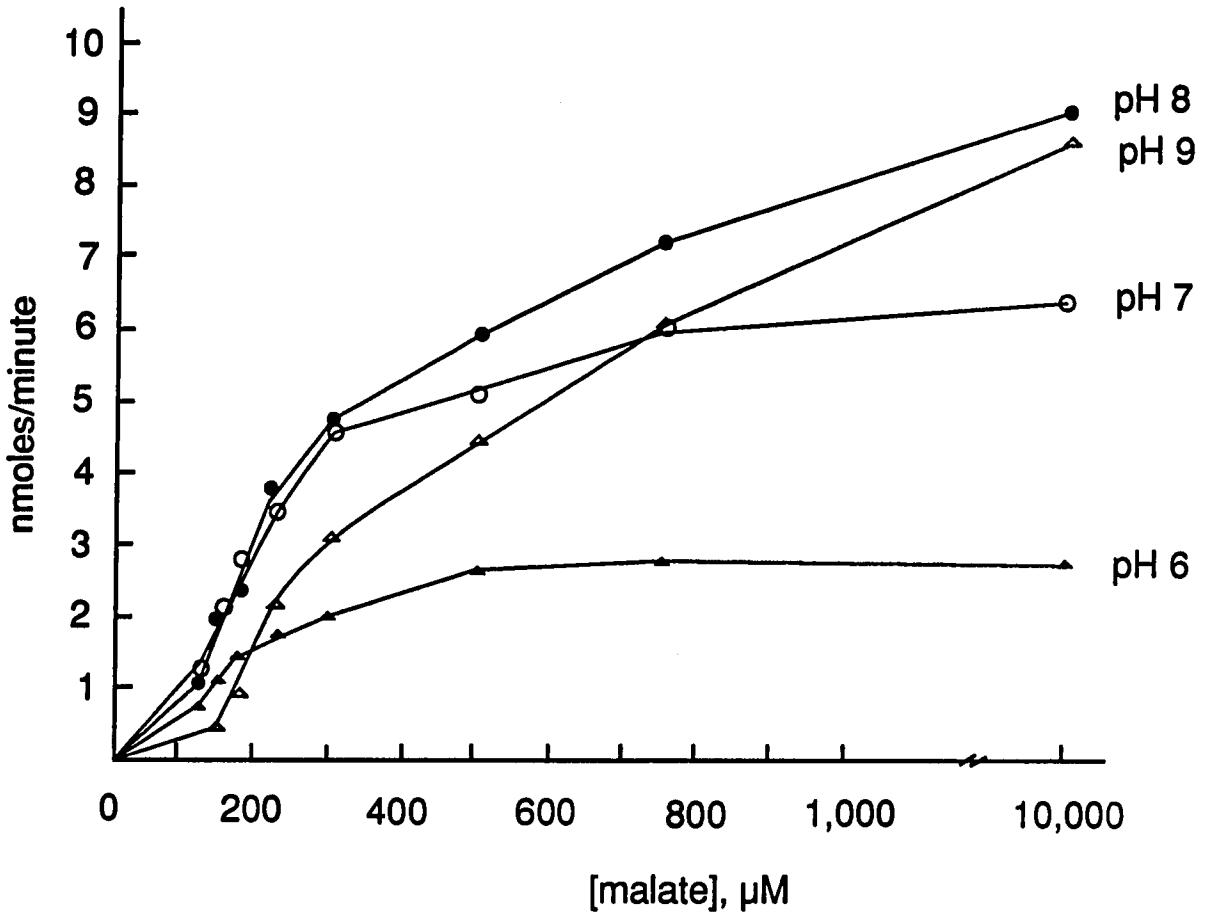

10
9
8
7
6
5
4
3
2
1
nmoles/minute
0
200
400
600
800
1,000
10,000
[malate], µM
pH 8
pH 9
pH 7
pH 6

Figure 5. Effect of Divalent Metal Cations on Gill mME Activity using the EDTA Buffereing System. The reaction mixtures contained 50 mM HEPES (pH 7), 10 mM sodium malate, 156 μM NADP, enzyme, and the concentrations of EDTA and metal ions indicated below.

Part A: Reaction mixtures contained 100 μM $MnCl_2$ and the indicated concentrations of EDTA.

Part B: Reaction mixtures contained 100 μM EDTA and the indicated amounts of $MnCl_2$.

Part C: Reaction mixtures contained 100 μM EDTA and the indicated amounts of $MgCl_2$.

Part D: Reaction mixtures contained 100 μM EDTA and the indicated amounts of $CoCl_2$.

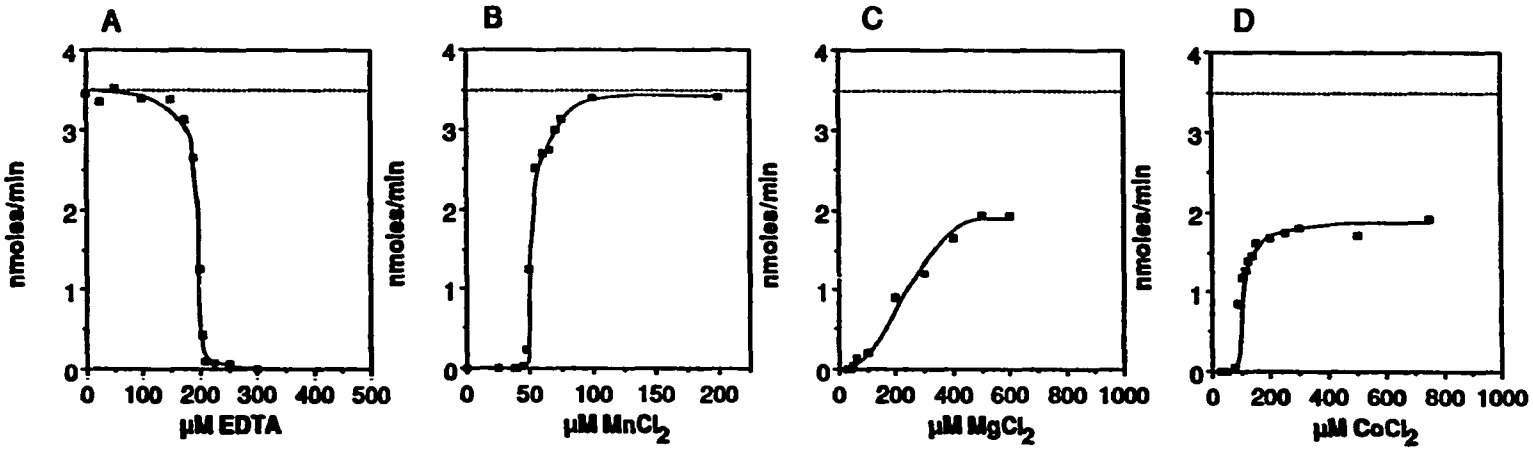
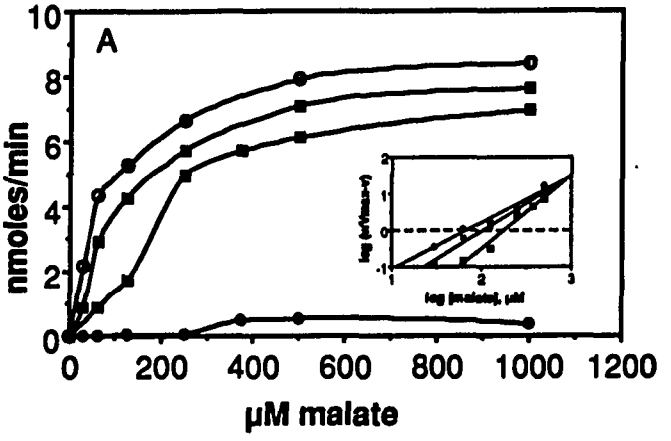
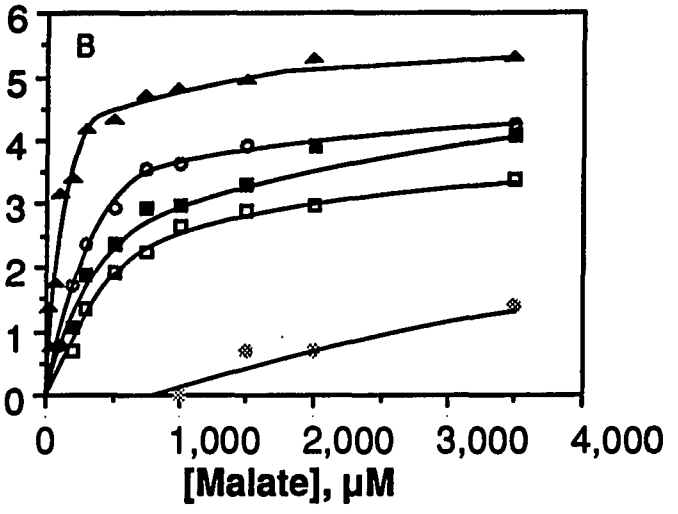

A
B
C
D
nmoles/min
μM EDTA
μM MnCl2
μM MgCl2
μM CoCl2
0
1
2
3
4
0 100 200 300 400 500
0 50 100 150 200
0 200 400 600 800 1000
0 200 400 600 800 1000

Figure 6. Effect of Increasing $MnCl_2$ Concentration on Gill mME Rectivity with Malate. Reaction mixtures contained 50 mM HEPES (pH 7.0), 156 μM NADP, enzyme, and the various concentrations of sodium malate and $MnCl_2$ concentrations indicated. Closed circles: 0 added $MnCl_2$. Open squares: 100 μM $MnCl_2$ added. Closed square: 200 μM $MnCl_2$ added. Open circles: 400 μM $MnCl_2$ added.

Figure 6B. Effect of Increasing $MnCl_2$ Concentration After Removal of Phosphate Ions. ME was dialyzed against 20 mM Tris pH 7, 0.5 mM DTT, and 20% glycerol. Closed circles: 0 added $MnCl_2$. Open squares: 100 mM $MnCl_2$. Closed squares: 200 μM $MnCl_2$. Open circles: 400 μM $MnCl_2$. Closed triangles: 1,000 μM $MnCl_2$.

Part C. Hill Plot of the 1,000, 400, and 100 μM $MnCl_2$ Curves Shown in Part B.


A
nmoles/min
µM malate
0
200
400
600
800
1000
1200
0
2
4
6
8
10


B
[Malate], µM
0
1,000
2,000
3,000
4,000
0
1
2
3
4
5
6

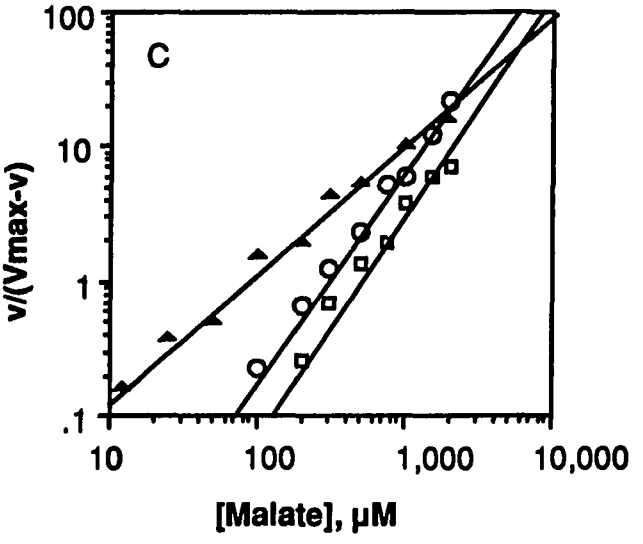
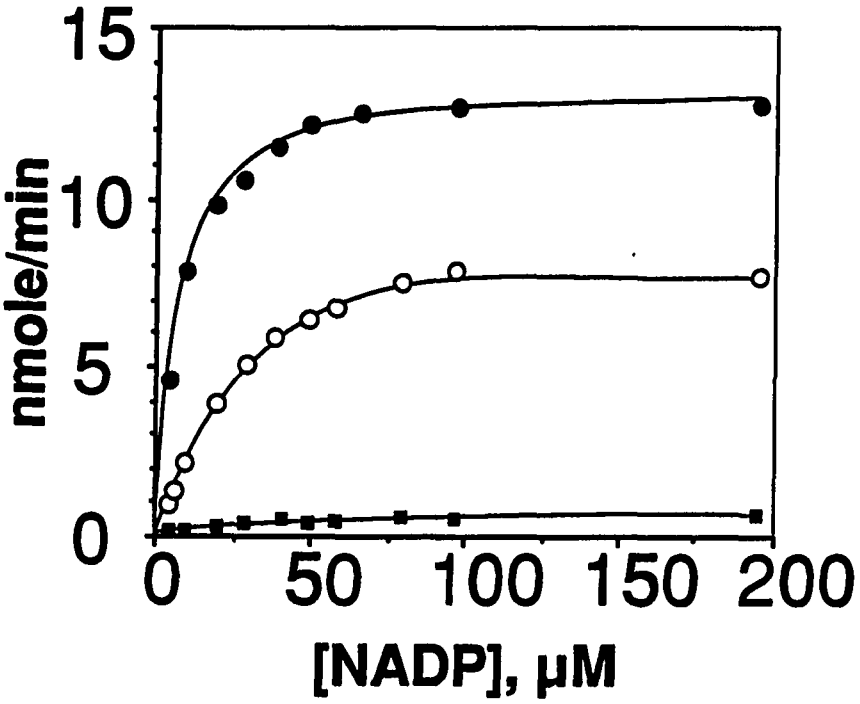

C
v/(Vmax-v)
[Malate], µM
10
100
1,000
10,000
.1
1
10
100

Figure 7. Effect of Increasing Concentrations of Malate on the NADP Utilization by mME from the Ribbed Mussel Gill Tissue. Reaction mixtures contained enzyme, 100 μM $MnCl_2$, 50 mM HEPES (pH 7), and the indicated concentrations of malate and NADP. Closed circles: 1,000 μM malate. Open circles: 300 μM malate. Closed squares: 80 μM malate.


15
10
5
0
nmole/min
0
50
100
150
200
[NADP], µM

malate increased with apparent $S_{0.5}$'s for malate of 179 μM, 218 μM, 319 μM, and 461 μM at pH's 6, 7, 8, and 9, respectively.

The dependency of enzyme activity on divalent cation concentration was determined (Fig. 5) for the partially purified gill mME. In the presence of 100 μM $MnCl_2$, malic enzyme activity could be inhibited by titration with EDTA (Fig. 5A). At 300 μM EDTA, no malic enzyme activity was detected. Conversely, in the presence of 100 μM EDTA, malic enzyme activity could be returned to the amount of activity observed in the absence of EDTA by titration with $MnCl_2$ (Fig. 5B). Under identical conditions, $MgCl_2$ and $CoCl_2$ could only restore 60% of the full enzyme activity (Fig. 5C and D). Estimates of the half saturated points on the curves (Fig. 5) for the metals in the presence of 100 μM EDTA were 92 μM, 50 μM, and 225 μM for Co^{2+}, Mn^{2+}, and Mg^{2+}, respectively. Analysis of the data with the "Chelate" correction for free divalent metal ion indicates that the gill mME has a relatively high $K_{0.5}$ for Mg^{2+} while very low $K_{0.5}$ values for Co^{2+} and Mn^{2+} were observed. These estimated values were Mn^{2+}: 18 picomolar; Co^{2+}: 1.1 picomolar; Mg^{2+}: 140 micromolar.

Confirmation of the estimated $K_{0.5}$ value for Mn^{2+} binding was made by the method of Flaschka (1959). Flaschka's method assumes that only the $EDTA^{4-}$ form binds metal, whereas at the pH at which these experiments were performed (pH 7), the majority of EDTA exists in the $EDTA^{3-}$ form. To account for the reduction in EDTA metal binding capability at pH 7, a pH correction factor was subtracted from the log stability constant. The log stability constant (13.79) for Mn^{2+} was reduced by 3.289 (correcting the log

stability constant to 10.501). The $K_{0.5}$ for Mn^{2+} by this method, 16 x 10^{-12} M for both the EDTA titration (Fig. 5A) and the metal ion titration (Fig. 5B), was in good agreement with that obtained with the "Chelate" program.

By increasing the concentration of $MnCl_2$ in the assay mixture at pH 7 from 100 to 400 μM, the apparent $S_{0.5}$'s for malate and the Hill coeffcients ("n" values) for malate rectivity with the mME were reduced (Fig. 6A). Hill coefficients of 2.00, 1.54, and 1.28 with apparent $S_{0.5}$ for malate of 178, 103, and 70 μM were obtained at Mn^{2+} concentrations of 100, 200, and 400 μM, respectively. As these assays were performed in the presence of phosphate ion (10 mM), the concentration of $MnCl_2$ added to the reaction mixture could not be increased above 400 μM without the formation of a visible precipitate. When the phosphate ion was removed from a similar enzyme preparation by dialysis against 3 20 x volume changes of a buffer composed of 20 mM Tris pH 7, 0.5 mM DTT and 20 % glycerol, the concentration of $MnCl_2$ could be increased without the formation of a visible precipitate. The results of these experiments are shown in Fig. 6B. Under these conditions, the $K_{0.5}$ for malate decreased from 0.445 mM to 0.398 mM to 0.270 mM to 0.079 mM as the $MnCl_2$ concentration was increased from 100 to 200 to 400 to 1,000 μM. Under these conditions, the "n" values were 1.42 at 100 μM, 1.49 at 400 μM, and 0.914 at 1,000 μM.

With 100 mM $MnCl_2$ at pH 7, as the malate concentration was increased from 80 to 300 to 1,000 μM, the apparent K_m for NADP decreased from 52 to 25 to 4 μM, respectively (Fig. 7).

All salts tested (NaCl, KCl, LiCl, and sodium acetate) inhibited the mME reaction (Fig. 8). Using these data, the following equation was used to determine the K_i for a competitive inhibitor with a very high K_i value:

$[I]_{50}=(1+[S]/K_m)(K_i)$.

With the apparent K_m for malate adjusted to 218 μM (as for conditions of 100 μM $MnCl_2$ in the presence of 10 mM sodium phosphate, pH 7.0), and the concentration of malate adjusted to 300 μM, the following K_i values were obtained: NaCl ($[I]_{0.5}$ = 80 mM) with a K_i =46 mM; KCl ($[I]_{0.5}$ = 65 mM) with a K_i = 38 mM; LiCl ($[I]_{0.5}$=40 mM) with a K_i = 23 mM; and sodium acetate ($[I]_{0.5}$ = 60 mM) with a K_i = 35 mM. All values were very similar indicating that the inhibition by these salts was not due to chloride or a specific alkali cation.

ATP did not affect malate binding when the enzyme was assayed in the presence of 10 mM $MnCl_2$ (Fig. 9). This metal ion concentration was chosen to circumvent possible problems with chelation of the metal ion cofactor by added ATP.

At pH 8, fumarate influenced malate binding at 5 mM in the presence of 10 mM $MnCl_2$ (Fig. 10), whereas succinate had little effect. From the Hill plot, 5 mM fumarate increased the malate K_m from 0.41 mM to 1.10 mM, and the Hill coefficient from 0.92 to 1.1 assuming that fumarate acted as a competitive inhibitor and did not decrease Vmax.

Table 2. Inhibition of the Ribbed Mussel Gill mME by CoA Derivatives.

ASSAY CONDITION[a]	OBSERVED VELOCITY
control	100%
+100 μM acetyl CoA	78.2%
+100 μM propionyl CoA	77.9%
+100 μM n-butyryl CoA	73.5%

[a]Reaction mixtures contained enzyme, 50 mM HEPES (pH 8), 2 mM $MnCl_2$, 156 μM NADP, 37.5 μM sodium malate, and the indicated concentrations of the additions.

Figure 8. Effect of Several Salts on the Ribbed Mussel Gill mME Reaction. Reaction mixtures contained enzyme, 50 mM HEPES (pH 7), 156 μM NADP, 300 μM malate, 100 μM $MnCl_2$, and the indicated concentrations of the various salts.

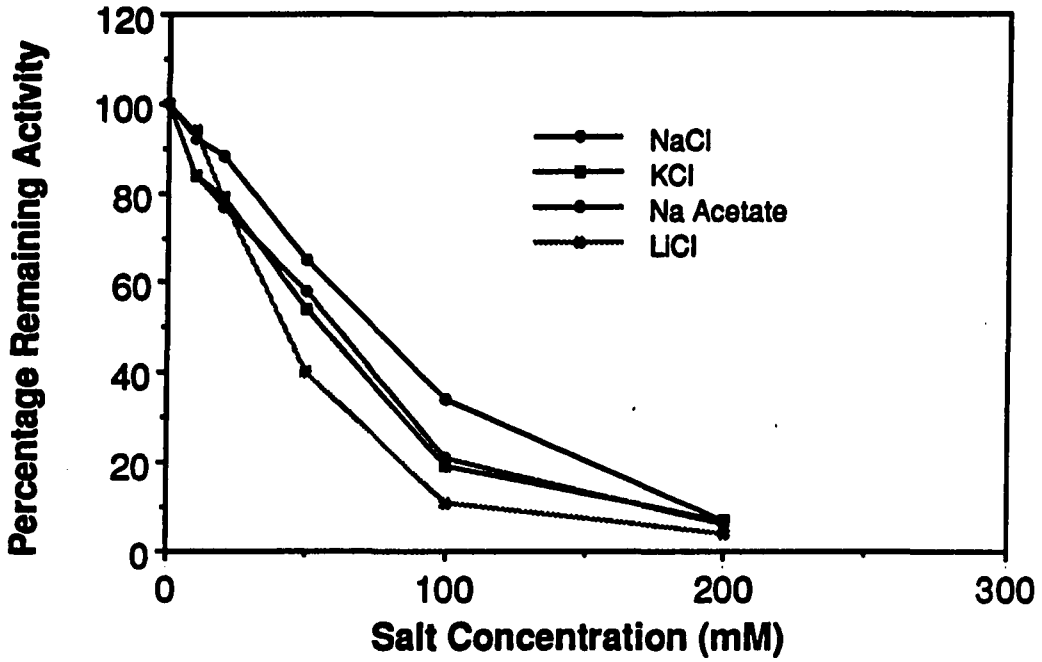

Percentage Remaining Activity
120
100
80
60
40
20
0
0
100
200
300
Salt Concentration (mM)
NaCl
KCl
Na Acetate
LiCl

Figure 9. ATP Has No Effect on Malate Binding to the Ribbed Mussel Gill mME. Reaction mixtures contained enzyme, 50 mM HEPES (pH 7), 10 mM $MnCl_2$, 156 μM NADP, and the various indicated concentrations of sodium malate and ATP. Closed circles: 0 mM ATP. Open circles: 1 mM ATP. Closed squares: 2 mM ATP. Open squares: 3 mM ATP.

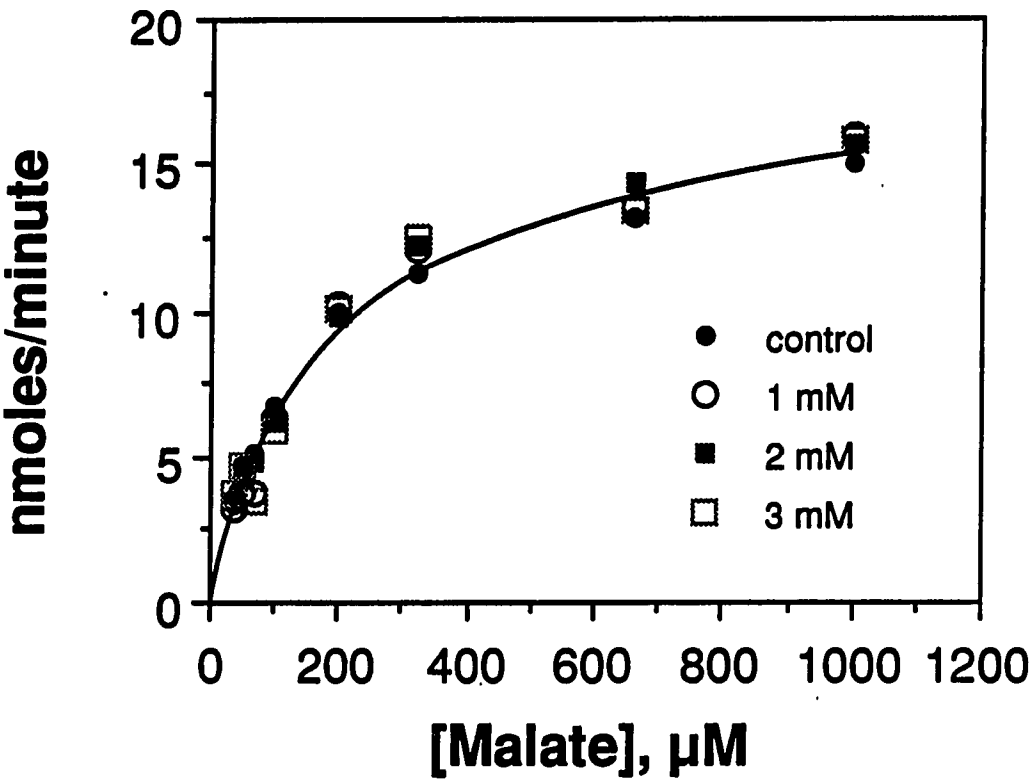
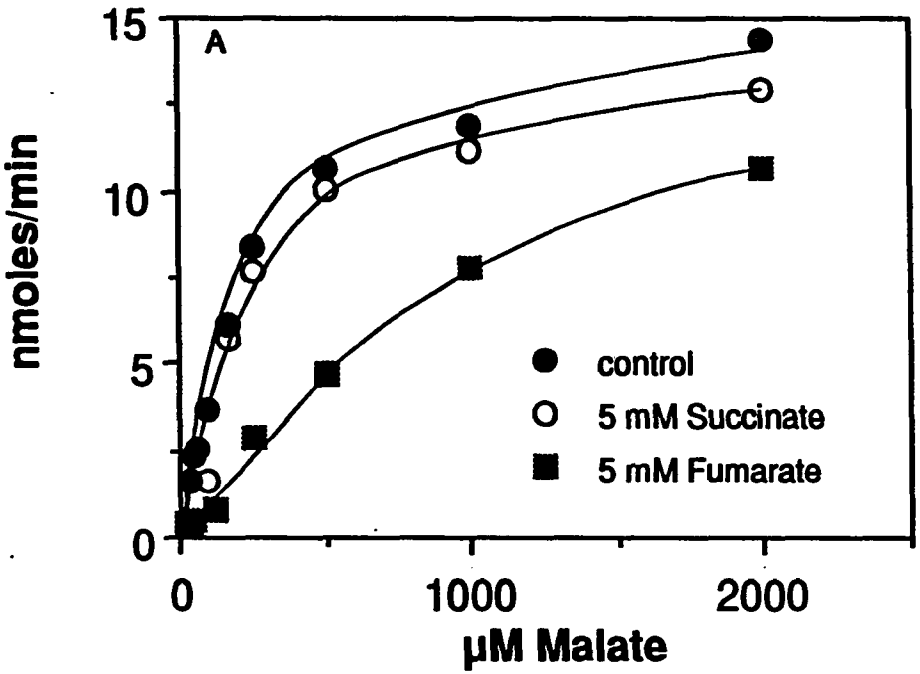

nmoles/minute
20
15
10
5
0
0
200
400
600
800
1000
1200
[Malate], µM
control
1 mM
2 mM
3 mM

Figure 10A. Effect of Succinate and Fumarate on the Reactivity of Malate with the Ribbed Mussel Gill Malic Enzyme. Reaction mixtures contained enzyme, 50 mM HEPES (pH 8), 156 μM NADP, 10 mM $MnCl_2$, and the indicated concentrations of sodium malate, sodium fumarate, and sodium succinate. Closed circles: control. Open circles: 5 mM succinate. Closed Squares: 5 mM fumarate.

Figure 10B. Hill Plot Showing the Effect of Fumarate. Open circles: control. Closed circles: 5 mM fumarate.


A
15
10
5
0
nmoles/min
0
1000
2000
µM Malate
control
5 mM Succinate
5 mM Fumarate

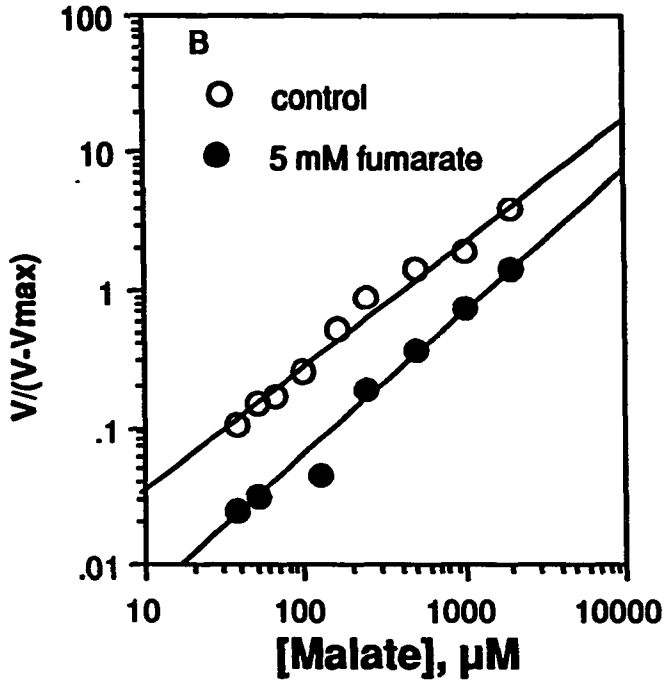
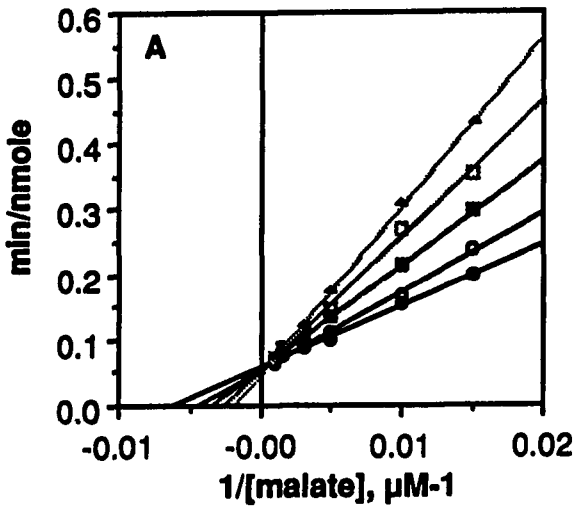

B
100
10
1
.1
.01
V/(V-Vmax)
10
100
1000
10000
[Malate], µM
control
5 mM fumarate

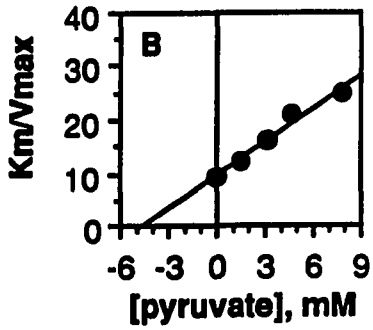
Figure 11 A. Pyruvate is a Competitive Inhibitor of Malate Utilization by the Ribbed Mussel Gill mME. Reaction mixtures contained enzyme, 50 mM HEPES (pH 7.0), 10 mM $MnCl_2$, 156 μM NADP, and the indicated quantities of malate and pyruvate. Closed circle: 0 mM pyruvate. Open circle: 1.57 mM pyruvate. Closed square: 3.15 mM pyruvate. Open square: 4.74 mM pyruvate. Closed triangle: 7.85 mM pyruvate.

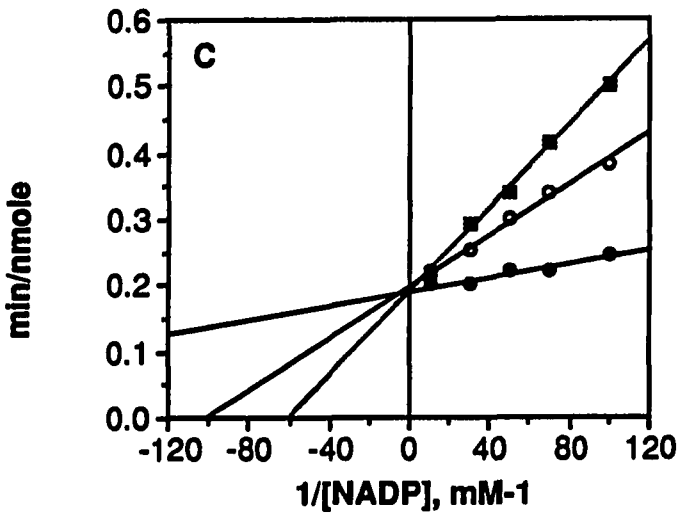
Figure 11 B. Slopes Calculated from Part A Plotted as a Function of Pyruvate Concentration.

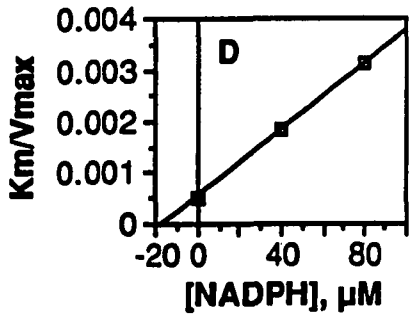
Figure 11 C. NADPH is a Competitive Inhibitor of NADP Utilization by the Ribbed Mussel Gill mME. Reaction mixtures contained enzyme, 50 mM HEPES (pH 7), 1 mM MnCl2, 10 mM malate, and the indicated quantities of NADP and NADPH. Closed circle: 0 μM NADPH. Open circle: 40 μM NADPH. Closed square: 80 μM NADPH.

Figure 11 D. Slopes Calculated from Part B Plotted as a Function of NADPH Concentration.


A
min/nmole
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-0.01
-0.00
0.01
0.02
1/[malate], µM-1


B
Km/Vmax
40
30
20
10
0
-6 -3 0 3 6 9
[pyruvate], mM


C
min/nmole
0.6
0.5
0.4
0.3
0.2
0.1
0.0
-120 -80 -40 0 40 80 120
1/[NADP], mM-1

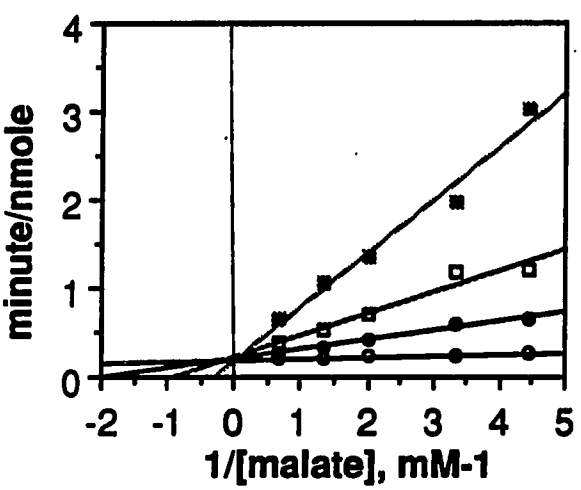

D
Km/Vmax
0.004
0.003
0.002
0.001
0
-20 0 40 80
[NADPH], µM

In an assay system using low concentration of malate (37.5 μM) in the presence of 2 mM $MnCl_2$, acetyl CoA, propionyl CoA, and n-butyryl CoA at 100 μM were inhibitory (Table 2).

Pyruvate was a competitive inhibitor of malate utilization (Fig. 11A). These assays were performed with 10 mM $MnCl_2$ to circumvent possible complications of metal ion binding by pyruvate. The replot of slope vs. inhibitor concentration was linear, and by extrapolation, the apparent K_i for pyruvate was 4.5 mM. NADPH was a competitive inhibitor of NADP utilization and the replot of slope vs. inhibitor concentration was linear (Fig. 11B). By extrapolation, the apparent K_i was 16 μM.

Hydroxymalonate inhibited malate utilization in a purely competitive fashion (Fig. 12). From the replot of slope vs. inhibitor concentration, the K_i for hydroxymalonate was determined to be 16 μM. Hydroxymalonate inhibited pyruvate utilization in a fashion consistent with non-competitive inhibition (Fig. 13). From the linear replot of slope as a function of hydroxymalonate concentration, K_i was estimated to be 147 μM, whereas the K_m for pyruvate under these conditions was estimated to be 4.7 mM.

Figure 12. Hydroxymalonate is a Competitive Inhibitor of Malate Utilization by the Ribbed Mussel Gill mME. Reaction mixtures contained enzyme, 50 mM HEPES (pH 7), 1 mM $MnCl_2$, 156 μM NADP, the indicated indicated concentrations of malate and hydroxymalonate. Open circles: 0 μM hydroxymalonate. Closed circles: 62.5 μM hydroxymalonate. Open squares: 187.5 μM hydroxymalonate. Closed squares: 500 μM hydroxymalonate.


minute/nmole
4
3
2
1
0
-2 -1 0 1 2 3 4 5
1/[malate], mM-1

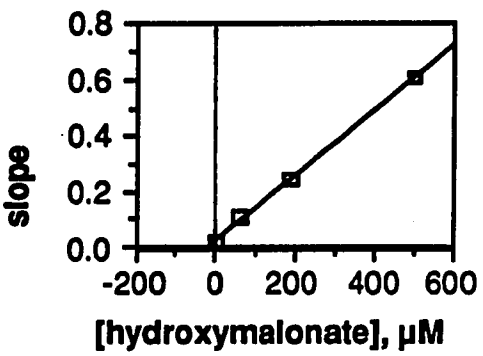
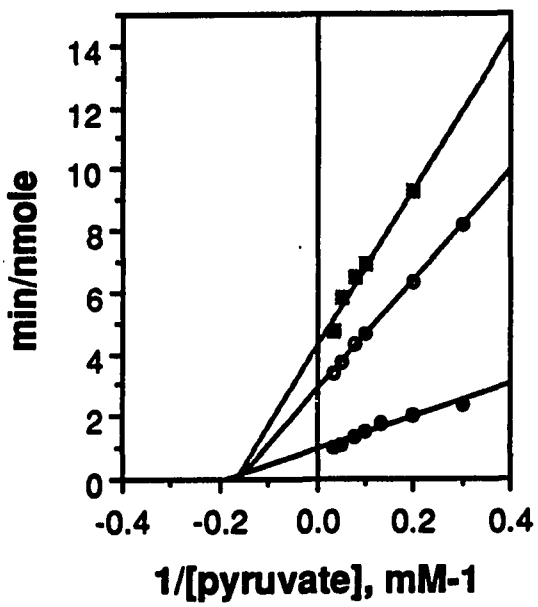
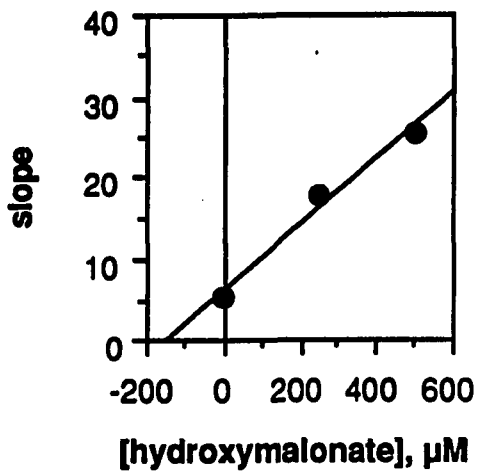

slope
0.8
0.6
0.4
0.2
0.0
-200 0 200 400 600
[hydroxymalonate], µM

Figure 13. Hydroxymalonate is a Non-Competitive Inhibitor of Pyruvate Utilization by the Ribbed Mussel Gill mME. Reaction mixtures contained 100 mM HEPES (pH 7), 1 mM $MnCl_2$, 100 mM sodium bicarbonate, 200 μM NADPH, and the various indicated quantities of pyruvate and hydroxymalonate. Closed circles: 0 mM hydroxymalonate. Open circles: 250 μM hydroxymalonate. Closed square: 500 μM hydroxymalonate.


min/nmole
14
12
10
8
6
4
2
0
-0.4
-0.2
0.0
0.2
0.4
1/[pyruvate], mM-1


slope
40
30
20
10
0
-200
0
200
400
600
[hydroxymalonate], µM

DISCUSSION

The native molecular weight for the ribbed mussel ME is similar to the native molecular weights M_r = 240,000 to 280,000 observed for tetrameric MEs from a number of animals, including the NAD-form from Ascaris (Fodge et al., 1972), the NADP-form from pigeon liver (Hsu and Lardy, 1967), and the NADP-form from rat liver (Saito et al., 1971). Lower molecular weight forms of the NADP-specific mME include the dimeric form from a tapeworm Hymenolepis diminuta with a M_r = 120,000 (Li et al., 1972), the tapeworm Taenia crassiceps with a M_r = 110,000 (Zenka and Prokopic, 1987), and the dimeric ME with M_r= 105,000 from the archaebacterium Solfonibus sulfataricus (Bartolucci et al., 1987). The small amount of NAD-ME-like activity eluting from the Sephadex column (Fig. 2) might be a smaller, minor form. Examples of mammalian tissue mitochondria possessing several ME forms as evidenced from the appearance of gel filtration chromatograms include: heart (Lin and Davis, 1974), adrenal cortex (Sauer, 1973), liver (Sauer, 1973), and skeletal muscle (Taroni et al., 1988). Native gel electrophoresis of whole gill tissue (Fig. 1) suggests that there are only three malate utilizing activities, however. These activities are the malic enzyme, the cytosolic malate dehydrogenases, and the mitochondrial malate dehydrogenases.

While there are several examples mMEs which use both NAD and NADP, such as those from rat adrenal cortex and liver (Sauer, 1973), tumors (Moreadith and Lehninger, 1984), canine intestinal mucosa (Nagel and Sauer, 1982), human skeltal muscle (Taroni et al., 1988) and cauliflower (Macrae and Moorhouse, 1970; Valenti and Pupillo, 1981), the mME from the ribbed

mussel gill appears to be fairly specific for NADP in that the rate of NAD reduction proceeds at only 2% the rate of NADP reduction (Fig. 2). The MEs from annelids, insects, molluscs, and other invertebrates seem to be similar to the mME from ribbed mussel gill tissue in their specificity for NADP(H) (Hoffmann et al., 1979; Hochachka and Mustafa, 1973; Norden and Matanganyidze, 1977; deZwaan, 1977; Storey et al., 1975).

The ME activity which elutes at M_r = 265,000 has by far the greatest amount of NADP-dependent malate oxidizing capability (Fig. 2). The MDH activity, when measured as oxaloacetate dependent NADH oxidizing capability is about 10 times more active than the ME, but as seen in the chromatogram, this activity is not very reversible.

The pH optimum for malate utilization by the gill mME (Fig. 3) is similar to that observed for the oyster muscle (Hochachka and Mustafa, 1973), for the silkworm blood ME (Faulkner, 1956), while the pH optimum of the pigeon liver cME is about 7.5 (Hsu, 1982). Inspection of the bell shaped curve suggests that amino acid residues with pK's of 6.5 and 10.5 may influence the malate dependent activity of this enzyme. These values indicate that the active site of this ME is probably similar in structure to MEs from other sources. However, the lowest apparent K_m's for malate are found at the lower more physiological pH's (Fig. 4).

The ribbed mussel gill mME is similar to other MEs in that it has an absolute requirement for divalent cations; EDTA inhibits ME activity (Fig. 5A). The addition of Mn^{2+} restores full activity, whereas other metal ions tested (Mg^{2+} and Co^{2+}) restore partial activity (Fig. 5B, C, and D). The NADP-

linked MEs generally use Mg^{2+}, Ni^{2+}, and Co^{2+} in addition to Mn^{2+} (Parvin et al., 1964; Massarini and Cazzulo, 1975; Milne and Cook, 1979). For example, the tsetse fly ME is activated by Mn^{2+}, Mg^{2+}, Co^{2+}, and Ni^{2+}, whereas Cd^{2+}, Ca^{2+}, and Zn^{2+} are ineffective (Norden and Matanganyidze, 1977).

By correcting for the free non-EDTA bound metal ions in solution, the metal ion affinities ($S_{0.5}$'s) of the ribbed mussel gill mME for Mn^{2+} and Co^{2+} are similar (18 picomolar and 2 picomolar) while the affinity for Mg^{2+} is much lower (140 μM). In the absence of EDTA and added metal ion, a small amount of activity is detectable, usually on the order of 1/10th the activity observed in the presence of 100 μM $MnCl_2$ (see Fig. 2). That this small amount of activity, (which can be totally inhibited with the addition of 100 μM EDTA) can be detected in the absence of added metal attests to the high affinity with which the enzyme binds metal, as it indicates the existence of metal-enzyme complexes even after extensive dialysis and gel filtration, consistent with the observation that ME exists as a metal-enzyme complex (Hsu et al., 1976). Mitochondrial Mn^{2+} appears to be entirely bound to some mitochondrial components (Azzone et al. 1976), if not to mitochondrial proteins then perhaps as manganese phophate (Bragadin et al., 1983). The values obtained for this ME using the "Chelate" program and Flaschka's formulas indicate that the tenacity of metal binding to this enzyme is much greater than that observed for MEs from various other sources. Previously reported values for metal binding $S_{0.5}$ values include: E.coli NADP-linked ME Mn^{2+} $S_{0.5}$ = 360 nM, Mg^{2+} $S_{0.5}$ = 54 μM (Brown and Cook, 1981); potato ME with NADP as cofactor Mn^{2+} $S_{0.5}$ = 110 μM (Grover et al., 1981); crayfish

NADP ME Mn^{2+} $S_{0.5}$ = 2.5 μM (Skorkowski et al., 1977); protozoan (Crithidia fasciculata) NADP-linked ME Mn^{2+} $S_{0.5}$ = 10 μM (Marr, 1973).

According to Schramm's criteria (1986) for the physiological dependency of the regulation of mammalian enzymes by Mn^{2+}, an enzyme must have an $S_{0.5}$ value of 100 nM or lower. While the ribbed mussel mME has an $S_{0.5}$ value which might qualify it for a candidate for Mn^{2+} regulation, the observation that the concentration of Mn^{2+} is much higher in the tissues of molluscs than in mammals (Simkiss and Mason, 1983) may make regulation by limiting Mn^{2+} unlikely. However, active mechanisms of Mn^{2+} ion regulation by molluscan mitochondria may occur, as have been described for mitochondria and other organelles in mammalian tissues (Ash and Schramm, 1982; Smeyers-Verbeke et al., 1977). Regulation of Mn^{2+} ion concentration at the organelle level may in turn regulate this enzyme. Although Mg^{2+} is less reactive than Mn^{2+}, the gill ME has a relatively low K_m for Mg^{2+}, so it may also be important physiologically. While the Mg^{2+} concentration in the mitochondria of molluscs has not beeen reported, the concentration of this metal ion free in the squid axoplasm has determined to be 2-3 mM (Baker and Crawford, 1972). The physiological importance of Co^{2+} in the regulation of this enzyme is not known.

Increased metal ion (Mn^{2+}) concentration in the reaction mixture lowers the apparent K_m for malate and decreases the degree of cooperativity for malate (Fig. 6). Increased malate affinity in the presence of increased metal ion is similar to the behavior reported for the pigeon liver enzyme (Hsu, 1982). Cooperativity with malate at high Mn^{2+} levels is observed at low

malate concentrations in MEs from sources such as the porcine heart mitochondrial ME (Lapis and Harrison, 1978), the freshly prepared oyster adductor muscle cME (Hochachka and Mustafa, 1973), the canine intestine mME (Nagel and Sauer, 1982), and the cytosolic and mitochondrial MEs from sea mussel adductor muscle (deZwaan, 1977) but not the squid mantle ME (Storey et al., 1975).

At low concentrations of $MnCl_2$, the gill mME shows hyperbolic kinetics with regard to NADP and increased affinity for NADP in the presence of increasing concentrations of malate (Fig. 7), a behavior which is similar to that observed for the NAD-linked Ascaris sp. enzyme which operates according to a random reaction mechanism (Landsperger et al., 1978). The apparent K_m's for NADP and malate at pH 7 with this gill ME are lower than those reported for the other molluscan MEs (deZwaan, 1977; Hochachka and Mustafa, 1973), but are similar to those in other animals (Hsu, 1982).

The several salts tested all inhibit gill ME (Fig. 7). The NADP-linked malic enzyme of E.coli is stimulated by low concentrations of K^+ and NH_4^+ (Sanwal and Smando, 1969), whereas concentrations of KCl greater than 0.1 M inhibit this enzyme causing sigmoidal kinetics with regard to malate (Spina et al., 1970). Stimulation of the MEs from Bacillus stearothermophilus and Clostridium thermocellus by K^+ and NH_4^+ has also been observed (Kobayashi et al., 1989; Lamed and Zeikus, 1981). Various potassium salts are inhibitory to pigeon liver ME with the K_i's in the 10 mM to 65 mM range (Schimerlik and Cleland, 1977). The K_i's of the various salts tested are similar to each other indicating the lack of a specific monovalent anion or cation effect. The

effect of salt may be physiologically important in bivalve molluscs because increased salinity of the bathing medium increases the chloride content of bivalve tissues (Sarkissian and Gomolinski, 1976). The possible role of salt in regulating this ribbed mussel gill ME activity with hypersaline stress in ribbed mussels is currently under investigation.

ATP inhibition of malic enzymes by competition for malate is a characteristic observed for several MEs, especially those associated with the mitochondria (rat adrenal cortex and liver mitochondrial: Sauer, 1973; tumor cell mitochondria: Moreadith and Lehninger, 1984; canine intestinal mucosa mitochondria: Nagel and Sauer, 1982; E. coli NAD-specific ME: Milne and Cook, 1979; NADP-specific and NAD-NADP linked from herring skeletal muscle: Skorkowski and Storey, 1988, 1990). This inhibition is not observed for the ribbed mussel mitochondrial ME when ATP up to 3 mM is included in the assay mixtures in the presence of 10 mM $MnCl_2$ (Fig. 9). For the frog egg mitochondrial ME, the basis of ATP inhibition is interference by this nucleotide with the metal ion binding site of the ME (Petrucci and Cesare, 1990). The increased metal ion concentration included in these assays may have prevented ATP's inhibitory effect. While the decrease in ATP concentration observed in the adductor muscle of the closely related M. edulis in response to whole organism aerial exposure has been shown to influence the activity of several enzymes (Ebberink and deZwaan, 1980), it seems that malate binding by the ribbed mussel gill mME is not regulated by this metabolite.

The various CoA derivatives (acetyl CoA, propionyl CoA, butyryl CoA) at the low concentration (100 μM) inhibited the enzyme about equally well (Table 2), suggesting that the CoA portion of these compounds might be the actual inhibitor. Malic enzymes have previously been reported to be stimulated and inhibited by CoA derivatives depending on the source and presumably the function of the enzyme. Typically, both mitochondrial and cytosolic forms are inhibited by acetyl CoA, with the mitochondrial form being the more sensitive (Frenkel, 1975). Low concentrations (0.1-0.5 mM) of acetyl CoA are also inhibitory to the E. coli ME (Sanwal et al., 1968). However, some plant MEs are actually stimulated by CoA derivatives. For example, the NAD-NADP-linked ME from cauliflower mitochondria is stimulated by 0.5 mM acetyl CoA (Day et al., 1984). The several CoA derviatives found to be inhibitory to the ribbed mussel gill mME may serve as intermediates in the anaerobic formation of volatile acids in this organism. However, because high concentrations CoA derivatives (100 μM) caused only 25% inhibition at a malate concentration well below the Km at the pH used (37 μM vs. 400 μM at pH 8), regulation of this ME by CoA derivatives may be relatively unimportant.

The effects of several dicarboxylic acids on ME activity is dependent on the enzyme source. We find that 5 mM fumarate is strongly inhibitory, causing the K_m for malate at pH 8 in the presence of 10 mM $MnCl_2$ to increase from 0.41 mM to 1.1 mM. The observed Hill coefficient ("n" value) also increases slightly from 0.92 to 1.1, suggesting a slight change in the cooperativity between subunits when fumarate is included (Fig. 10A and B). Succinate inhibits malate binding slightly. These results indicate that this enzyme is

unlike the many mitochondrial mMEs studied previously Whereas fumarate and succinate are competitive inhibitors of the cME from pigeon liver (Schimerlik and Cleland, 1977), these compounds are stimulatory to many mMEs. Specifically, succinate is stimulatory to the mMEs from rat adrenal cortex and liver (Sauer, 1973) and crayfish abdomenal muscle (Swierczynski et al., 1980). Fumarate is stimulatory to the mMEs from herring skeletal muscle (Skorkowski and Storey, 1988, 1990), canine intestinal mucosa (Nagel and Sauer, 1982), mammalian tumors (Moreadith and Lehninger, 1984), rat adrenal cortex and liver (Sauer, 1973), Ascaris muscle (Landsperger and Harris, 1976), and crayfish abdomenal muscle (Swierczynski et al., 1980). The porcine heart mME is unique in that succinate is stimulatory while fumarate is inhibitory (Lapis and Harrison, 1978). As with fumarate and succinate, aspartate stimulates some MEs including those from E. coli (Milne and Cook, 1979), Neurospora crassa (Zink, 1967), porcine heart mitochondria (Lapis and Harrison, 1978). Oxaloacetate is inhibitory toward several MEs from several sources including the blue mussel adductor muscle (deZwaan, 1977), tsetse fly (Norden and Matanganyidze, 1977), and E. coli (Sanwal and Smando, 1969).

The relative lack of inhibition of malate oxidation in the presence of succinate may permit the operation of this enzyme under anaerobic conditions when succinate is observed to accumulate (Ho and Zubkoff, 1982). While the increased concentration of succinate in the ribbed mussel appearing in response to anaerobiosis has been noted by Ho and Zubkoff, the concentration of fumarate has not been reported. However, fumarate does not accumulate to as great an extent as succinate in response to anaerobiosis

when malate is supplied to isolated mitochondria from the closely related bivalve M. edulis (deZwaan et al., 1981). Similarly, succinate accumulates to a much greater extent than fumarate when malate is supplied to isolated mitochondria from Tubifex sp. (Schöttler, 1977a, b). Because of the lack of accumulation, inhibition of this ME by fumarate is probably not physiologically important.

The pattern of product inhibition is diagnostic of the reaction mechanism of an enzyme. NADPH is a competitive inhibitor for NADP for all MEs examined thus far, as it is for the ribbed mussel gill mME (Fig. 11). Pyruvate is a competitive inhibitor of malate for the randomly operating NAD-ME from Ascaris sp.(Landsperger et al., 1978; Park et al., 1984), but is uncompetitive for the ordered NADP-ME from pigeon liver (Chang and Hsu, 1973), suggesting the release of CO_2 prior to the release of pyruvate (Hsu et al., 1967) for the pigeon liver enzyme. Pyruvate is a competitive inhibitor of malate for this ribbed mussel gill mitochondrial ME with an apparent K_i of 4.5 mM (Fig. 11A). This result is similar to the result obtained for the crayfish NADP-ME for which the pyruvate K_i is 5.4 mM (Skorkowski et al., 1977). These results may indicate that like the Ascaris mME, the crayfish mME and the ribbed mussel mME all operate according to a random reaction mechanism. In support of the random reaction mechanism, $MnCl_2$ concentration influences malate binding (Fig. 5), and malate concentration influences NADP binding (Fig. 6).

Malate serves as an anaerobic fuel for the mitochondria of bivalves from several species (Burcham et al., 1984; Ballantyne and Moon, 1985; deZwaan et

al., 1981). The isolated mitochondria of the ribbed mussel lack the pyruvate sparking of malate utilization observed for mammalian mitochondria (Burcham et al., 1984), a behavior which may indicate the importance of the mME in pyruvate synthesis (Skorkowski et al., 1984). Hydroxymalonate, shown to be inhibitor of MEs from many sources, may be useful in demonstrating the importance of ME in the synthesis of alanine and acetyl CoA from malate. For the ribbed musel gill mME, hydroxymalonate is a competitive inhibitor of malate (Fig. 12), with a K_i of 16 μM. This apparent K_i is considerably lower than that observed for malate utilization by MEs from other sources, including the NAD-ME from Ascaris suum (apparent K_i=300 μM: Landsperger et al., 1978) which operates according to a random reaction mechanism (Park et al., 1986) and the ordered NADP-ME from pigeon liver (apparent K_i= 180 μM: Schimerlik and Cleland, 1977). Hydroxymalonate is a non-competitve inhibitor of pyruvate for the ribbed mussel mME (Fig. 13) with the apparent K_i=147 μM. Similarly, hydroxymalonate is a non-competitive inhibitor of the pigeon liver cME with an apparent K_i of 1.3 mM (Schimerlik and Cleland, 1977). Because of the apparent high affinity of the ribbed mussel ME for hydroxymalonate, this inhibitor should prove useful in determining the physiological role of the ribbed mussel gill mME.

While the specific activity of malic enzyme is low in this tissue, the rate of pyruvate formation is sufficient to account for the rate of alanine produced in response to hyperosmotic stress. Specifically, 31 grams of starting material has the ability to produce 9.38 μmoles pyruvate per minute, or 18.15 μmoles pyruvate/hour/gram wet weight. Assuming that each gram wet is equivalent to 0.1 gram dry, the rate of pyruvate formation is then 182 μmoles

pyruvate/hour/gram dry. With the alanine concentration increasing from 10 μmoles/gram dry weight to 150 μmoles/gram dry weight in four hours, the ME could be inhibited by greater than 70% and still produce enough pyruvate to account for alanine production.

The physiological role of the ribbed mussel gill ME therefore appears to be in the synthesis of the three carbon precursor of alanine and acetate (pyruvate) from a four carbon precursor (malate), while possibly providing reduced nucleotides for the production of succinate via the NADH-linked fumarate reductase reaction (Holwerda and deZwaan, 1980). A similar series of reactions is postulated to occur in Tubifex sp. (Hoffmann et al., 1979). In parasitic helminths which possess high activity NADP-linked MEs, a transhydrogenase (NADPH-NAD) activity is also observed (Hymenolepis diminuta: Fioravanti, 1982; Fasciola giganta: Umezurike and Anya, 1981) associated with the inner mitochondrial membrane (McKelvey and Fioravanti, 1985). A transhydrogenase may be indicated in the mitochondria of the ribbed mussel by the apparent specificity of this ME for NADP. Interestingly, the malic enzyme of Ascaris suum, like the malic enzyme of the ribbed mussel, operates by a random kinetic mechanism. These organisms have in common the anaerobic production of succinate, and it is likely that all malic enzymes which produce reduced nucleotides for succinate production operate by such a reaction mechanism.

CHAPTER 5.

PURIFICATION OF THE MALIC ENZYME FROM RIBBED MUSSEL (MODIOLUS DEMISSUS) GILL TISSUE MITOCHONDRIA

Abstract: NADP-Malic enzyme (ME) activity was purified from ribbed mussel gill tissue mitochondria and found to be a tetramer composed of subunits with M_r = 65,000. At pH 7, with 1 mM $MnCl_2$, Michaelis-Menton kinetics were observed with malate and NADP with apparent K_m's of 43 μM and 4 μM, respectively. The apparent K_m's for NADPH, pyruvate, and sodium bicarbonate were 129 μM, 8.5 mM, and 19 mM, respectively. NADPH and pyruvate exhibited substrate inhibition at high concentrations (NADPH > 100 μM; pyruvate > 20 mM). ATP was a mixed competitive inhibitor of NADP (K_i=0.51 mM). While the enzyme reaction is freely reversible when substrate concentrations are optimized, reversibility in vivo is probabaly limited by low tissue bicarbonate (CO_2) and pyruvate concentrations. Therefore, the physiological direction of the enzyme is the production of pyruvate, CO_2, and NADPH from malate and NADP.

INTRODUCTION

Malic enzyme (ME) activity and cellular localization have been described in the tissues of a number of vertebrate and invertebrate organisms. Mammalian heart muscle (Frenkel, 1972), brain (Salganicoff and Koepper, 1968), adrenal cortex (Brdiczka and Pette, 1971; Simpson and Estabrook, 1968, 1969b) possess cytosolic and mitochondrial forms of ME, while adipose (Frenkel, 1972) and adrenal medulla (Brdiczka and Pette, 1971) have only a cytosolic form. The majority of ME from avian and mammalian liver is cytosolic (Utter, 1959; Brdiczka and Pette, 1971). The cytosolic and mitochondrial forms from mammalian tissues are distinct gene products as they have different mobilities on non-denaturing starch gels (Henderson, 1966), can be resolved by anion exchange chromatography (Frenkel, 1972), are immunologically distinct (Isohashi et al., 1971; Bartholomé et al., 1972), show differential sensitivity toward sulfhydryl reagents (Frenkel and Cobo-Frenkel, 1973) and show differences kinetic behavior (Schimerlik and Cleland, 1977a; Frenkel and Cobo-Frenkel, 1973; Simpson and Estabrook, 1969a).

Several functions for MEs have been postulated based on the cellular distribution and kinetics. These include the use of malate for NADPH generation for steroid biosynthesis in adrenal cortex mitochondria (Simpson and Estabrook, 1968, 1969a and b) for cytosolic fatty acid and lipid biosynthesis (Young et al., 1964) and possibly for cytosolic gluconeogenesis in the livers of some vertebrate species (see Frenkel, 1975).

ME kinetic characteristics and cellular localization have also been described in several invertebrates with the NAD-linked ME of the parasitic helminthes Ascaris suum (round worm) and the NADP-linked ME of Hymenolepis sp. (tapeworm) among the best studied of the invertebrate MEs (Fodge et al., 1972; Fioravanti, 1982). The MEs from these parasitic helminths are mitochondrial and the reduced nucleotides generated by these MEs drive the co-localized fumarate reductase for the anaerobic synthesis of succinate (McKelvey and Fioravanti, 1985). A very active ME is found in the mitochondria of abdomenal muscles of crayfish (Skorkowski et al., 1977; Swierczynski et al., 1980).

ME activity has been observed in the tissues of molluscs. In the sea mussel (Mytilus edulis), ME is distributed approximately equally between the cytosolic and mitochondrial compartments of adductor muscle and gill while it is primarily cytosolic in the mantle and hepatopancreas (deZwaan and vanMarrewijk, 1973; Paynter et al., 1985a). In the oyster adductor muscle, ME is primarily cytosolic (Hochachka and Mustafa, 1973). In the gill tissue of ribbed mussels, oysters and quahogs, ME is primarily mitochondrial (Paynter et al., 1985a).

The kinetic characteristics of MEs from several molluscan species have been studied in a few impure preparation, including: oyster muscle (Hochachka and Mustafa, 1973), sea mussel mantle and muscle (deZwaan, 1977; deZwaan and vanMarrewijk, 1973), and squid mantle muscle (Storey et al., 1975). In these studies with molluscan MEs, both mME and cME forms

show some cooperativity with regard to malate and require a divalent cation for activity.

Malate is an anaerobic fuel for molluscan mitochondria (deZwaan et al., 1981). Malate transported into the mitochondria is distributed into many of the products which are observed to accumulate during anaerobic stress in tissues of the sea mussel, especially succinate and proponiate. Like the sea mussel, the ribbed mussel synthesizes propionate and succinate in response to anaerobic stress; however, higher concentrations of alanine accumulate in the tissues of the ribbed mussel in response to anaerobic stress (Ho and Zubkoff, 1982). Exposure of the ribbed mussel to hyperosmotic stress also results in the synthesis of larger concentrations of alanine (Baginski and Pierce, 1977; Greenwalt and Bishop, 1980) than are observed in the sea mussel in response to hyperosmotic stress (Livingstone et al., 1979). While the activity which synthesizes alanine, the alanine aminotransferase, is solely mitochondrial in the ribbed mussel, this activity has both cytosolic and mitochondrial forms in the sea mussel (Paynter et al., 1985a). Because of the existence of alanine aminotransferase in both cell compartments, there may be futile cycling of alanine or synthesis of alanine at a reduced rate in the sea mussel. This may account for the small amount of alanine which accumulates in sea mussel tissues compared to ribbed mussel tissues.

The possible use of malate as a precursor for mitochondrial pyruvate synthesis has been preposed because isolated mussel mitochondria may not transport pyruvate into the mitochondria for coupled respiration (Burcham et al., 1984; Ballantyne and Moon, 1985) even though they have a fully

functional pyruvate dehydrogenase (Paynter et al., 1985b). The co-localization of ME and alanine aminotransferase in the mitochondria of the ribbed mussel gill tissue means that production of pyruvate from malate and subsequent conversion to alanine could be coupled.

In this paper, the purification and properties of the ribbed mussel gill mitochondrial ME are described.

MATERIALS AND METHODS

Animals: Ribbed mussels (Modiolus demissus) were purchased from Northeast Environmental Laboratories (Monument Beach, MA) and were maintained as described by Greenwalt and Bishop (1980).

Chemicals and Reagents: All reagents were purchased from Sigma Chemical Company (St., Louis, Mo.) with the following exceptions: ammonium sulfate (enzyme grade) was purchased from Schwartz-Mann, Orangeberg, N.Y.; ATP agarose (C-6 linked) was purchased from Pharmacia; $MnCl_2$ was purchased from J.T. Baker Co., Phillipsburg, N.J. Miracloth was purchased from Calbiochem.

Enzyme Assay: All assays were performed at 23°C. One unit of activity is that amount of enzyme catalyzing the formation of 1 μmole product per minute. During purification, ME activity was monitored by measuring NADP reduction on a Beckman 3600 recording spectrophotmeter in a 2.0 ml reaction mixture consisting of 50 mM HEPES (pH 8), 10 mM sodium malate, 156 μM NADP, 100 μM $MnCl_2$ and enzyme.

Kinetics with regard to malate and NADP were examined in 2.0 ml reaction mixtures with purified enzyme, 1 mM $MnCl_2$, and 50 mM HEPES (pH 7) and the concentrations of malate and NADP indicated in the results section. Kinetics with regard to pyruvate, NADPH, and sodium bicarbonate were also performed in 2.0 ml reaction mixtures with purified enzyme, 1 mM $MnCl_2$, HEPES (100 mM, pH 7), and the concentrations of pyruvate, NADPH, and sodium bicarbonate indicated in the results section.

Protein concentrations were determined by Miller's (1959) modification of the Lowry method using bovine serum albumen as a standard. Spector's (1978) modification of Bradford's method was used for purified preparations when glycerol was added to the buffers.

Enzyme Purification (Table 1): All procedures were performed at 2-4°C. Ribbed mussel gills (from about 90 animals) were rapidly removed and weighed then homogenized twice in 10 volume/weight of ice cold homogenization buffer (0.4 M sucrose, 20 mM K-HEPES, and 1 mM K-EGTA, pH 7.5) using an Ultra-turax set at "45" for 10 seconds. The homogenate was filtered through one layer of Miracloth, then centrifuged at 1,500xg for 8 minutes. The supernatant was centrifuged at 9,000xg for 15 minutes. The supernatant from this step (denoted cytosol) was frozen for further study; the pellet contained mitochondria (Burcham et al., 1984).

The mitochondria containing pellet was resuspended in ice cold 100 mM sodium phosphate, pH 6.8, and sonicated using a Branson Sonic Power sonicator with 1.5 cm diameter probe set on "8" three times for 20 seconds each with 5 second intervals. Powdered ammonium sulfate was added to the sonicate to a concentration of 30% saturation with constant stirring. The mixture was centrifuged for 20 minutes at 15,000xg and the supernatant was then brought to 70 % saturation by the addition of powdered ammonium sulfate and again stirred for 1 hour at 4°C. The pellet from the 30-70% ammonium sulfate precipitation was resuspended in 100 mM sodium phosphate buffer and was dialyzed against the same buffer. The dissolved pellet was applied to a Sephadex G-150 column (36 cm x 4.5 cm). Active

fractions were made 50 mM sodium phosphate with the addition of an equal volume of H_20 and applied to a DEAE cellulose column (1.5 x 24 cm), equilibrated in 20 mM sodium phosphate (pH 7) and washed with 100 ml of the same buffer, then the ME activity was eluted with a linear KCl gradient in the same buffer (Fig. 1). Active fractions were pooled and dialyzed against 20 mM sodium phosphate buffer, then applied to a Reactive Red (Sigma Chemical Co.) column (1 cm x 16 cm) equilibrated in the same buffer. The column was washed with the same buffer and ME activity eluted with a 0-1 M KCl gradient in the same buffer (Fig. 2). Fractions containing ME activity were pooled and dialyzed against a buffer containing 20 mM Tris (pH 7), 20% glycerol, and 0.5 mM DTT (purification buffer).

The remaining purification step was modified after Moreadith and Lehninger (1984). Following dialysis, the preparation from the Reactive Red column was mixed with an equal volume of 2 mM sodium fumarate plus 2 mM fumarate in purification buffer and applied to a 5 ml ATP agarose column equilibrated in the same buffer. The column was washed with the purification buffer supplemented with 1 mM fumarate and 1 mM $MnCl_2$, then this mixture supplemented with 0.2 M KCl. ME activity was then specifically eluted with the fumarate, $MnCl_2$, and KCl mixture supplemented with 2 mM NADP (Fig. 3). The ME activity would not adsorb to the column when fumarate and $MnCl_2$ were omitted. Succinate would not substitute for fumarate. The purified enzyme was dialyzed against purification buffer to remove fumarate and NADP, then stored at -20°C in small aliquots which were defrosted just prior to use in kinetic studies.

Native Gel Electrophoresis: Purified protein was applied to a 7 % polyacrylamide slab gel using Tris at pH 8.8 as the gel buffer and Tris glycine as the running buffer and electrophoresed at 35 milliamps for 6 hours at 3°C at which time the bromophenol blue marker had reached the bottom of the gel (Fig. 4). Half of the gel was stained for malic enzyme activity as described by Harris and Hopkinson (1976) , while the other half was stained with silver according to Oakley et al. (1980).

SDS Polyacrylamide Gel Electrophoresis: The purified enzyme was subjected to one dimensional polyacrylamide slab gel electrophoresis in the presence of SDS according to the method of Laemmli (1970). Approximately 7 µg of purified enzyme (Bradford method) were exhaustivley dialyzed against ddH_2O to remove buffer, and then lypholyzed. The powder was dissolved in 50 µl 2 x Laemmli sample buffer, heated for 2 minutes at 110°C, cooled and electrophoresed on a 12.5% polyacrylamide gel for 4.5 hours at 35 milliamps (Fig. 4).

Kinetics: The assay mixtures for studying malate utilization or formation are described with the results. The reactions were initiated by the addition of the purified enzyme in the purification buffer. Data from the substrate reactivity experiments were analyzed using Cleland's (1979) computer program.

RESULTS

Malic enzyme activity elutes as a single symmetrical peak from Sephadex (see Chapter 4). The purified mME activity eluted as a peak with a leading edge from the DEAE column (Fig. 1) and a single symmetrical peak from a reactive red column (Fig. 2). The enzyme adhered to ATP agarose in the presence of fumarate and $MnCl_2$ (Fig. 3). The four column purification procedure produced a 200 fold purification of this ME (Table 1). Electrophoresis of the purified ME on native polyacryalmide gels produced a single silver staining band that reduced NADP in the presence of malate and $MgCl_2$ (Fig. 4, lanes A and B). Electrophoresis in the presence of SDS yielded a single Coomassie Blue staining band with an apparent M_r = 65,000 (Fig. 4C).

Kinetics: Previous studies with partially purified mME indicated that the optimal pH for malate utilization and malate synthesis were pH 8.5 and pH 6, respectively (Chapter 4). However, the apparent K_m for malate was lowest at pH 6-7, and reactivity of the enzyme with malate was increased as the $MnCl_2$ concentration was increased.

Therefore, the studies reported here were performed with 1 mM $MnCl_2$ at pH 7.0. Under these conditions, reactivity with malate and NADP showed hyperbolic kinetic patterns (Figs. 5 and 6) with apparent K_m values of 43 μM and 4 μM, respectively. When assayed in the reverse direction, the enzyme showed substrate inhibition at high levels of pyruvate and NADPH (Figs. 7 and 8). Using Cleland's computer program for the non-inhibitory portions of

Figure 1. Elution of Ribbed Mussel ME from DEAE Cellulose. See Materials and Methods for conditions. Open squares indicate ME activity measured in each fraction, and closed symbols indicate the concentration of KCl in each fraction determined by fraction conductivity. Each fraction contained 5.25 ml.

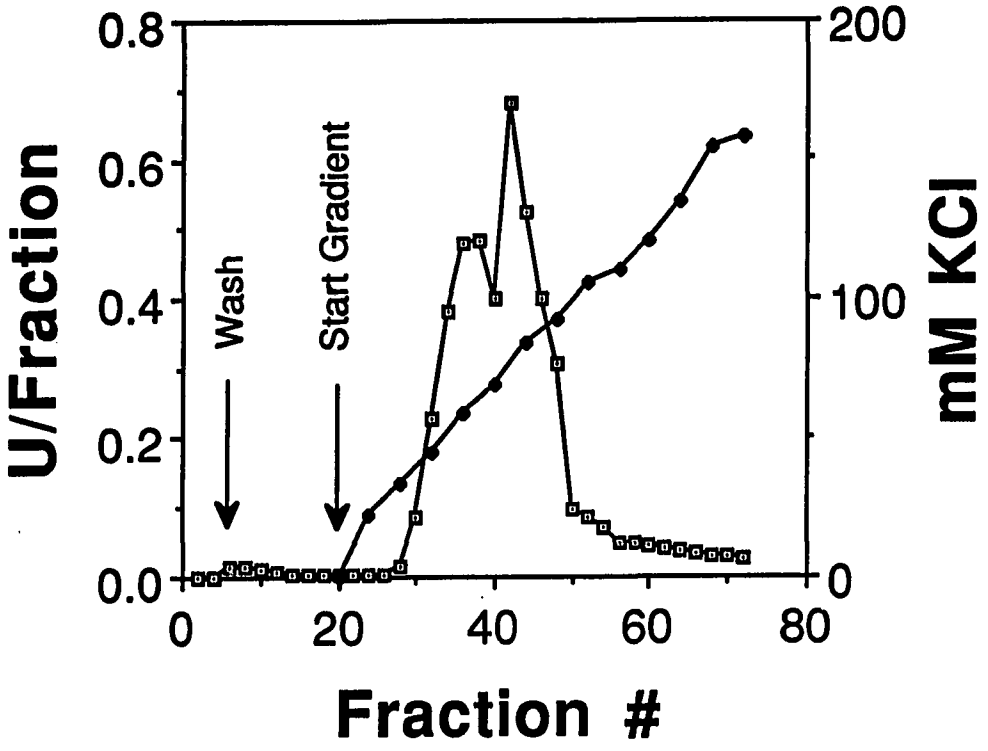

0.8
0.6
0.4
0.2
0.0
U/Fraction
Wash
Start Gradient
200
100
0
mM KCl
0
20
40
60
80
Fraction #

Figure 2. Reactive Red Agarose Chromatography of Ribbed Mussel ME. See Materials and Methods for conditions of operation. Open squares indicate ME activity and closed symbols indicate the concentration of KCl in each fraction determined by fraction conductivity. Each fraction contained 5.25 ml.

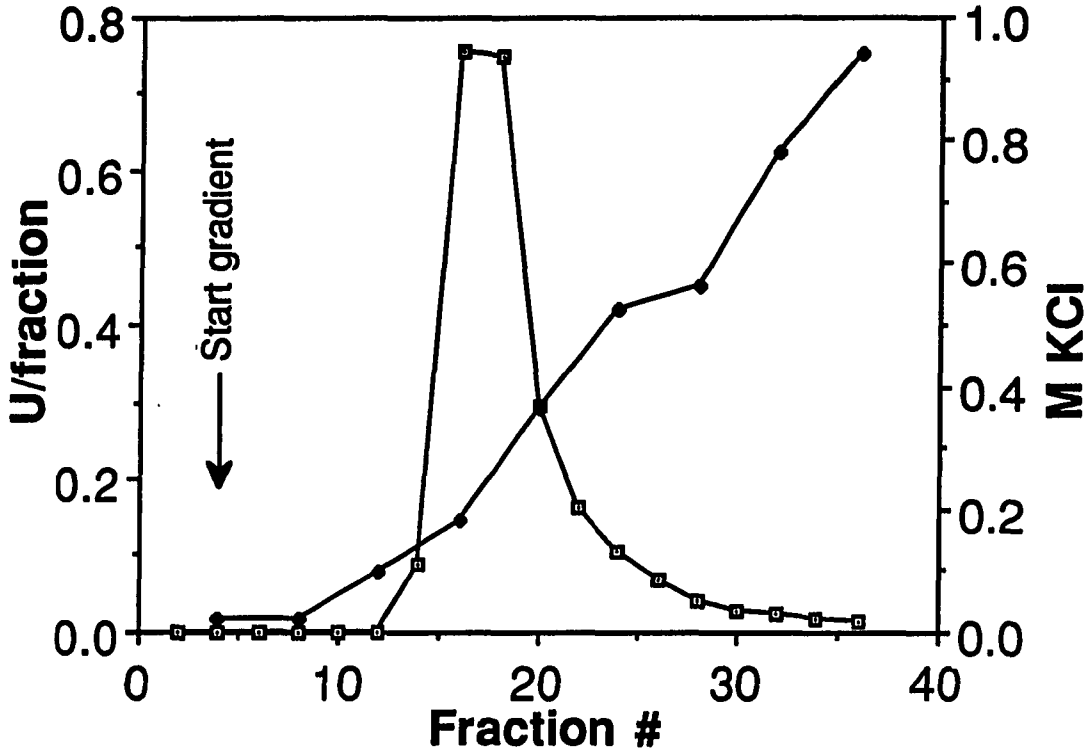

0.8
0.6
0.4
0.2
0.0
U/fraction
Start gradient
1.0
0.8
0.6
0.4
0.2
0.0
M KCl
0
10
20
30
40
Fraction #

Figure 3. Chromatography of Ribbed Mussel ME on ATP Agarose. See Materials and Methods for conditions of operation. Each fraction contained 5.25 ml.

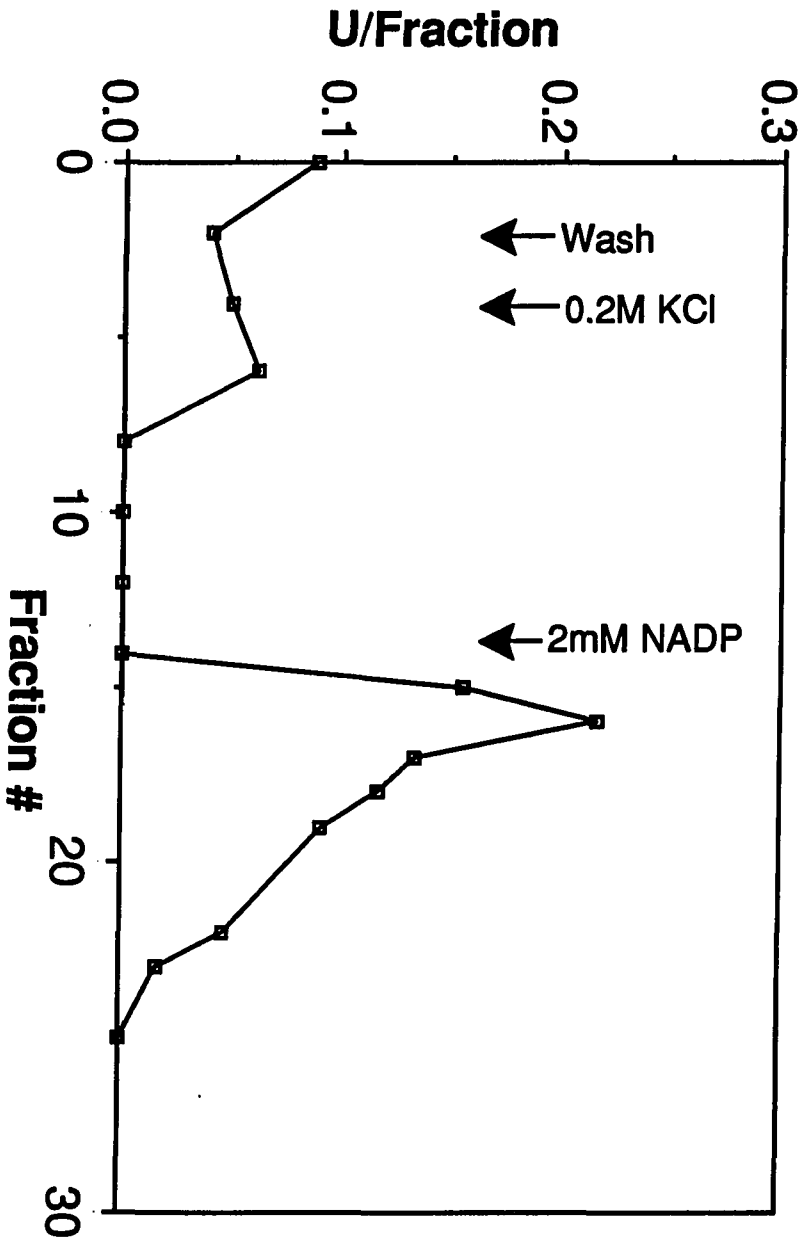
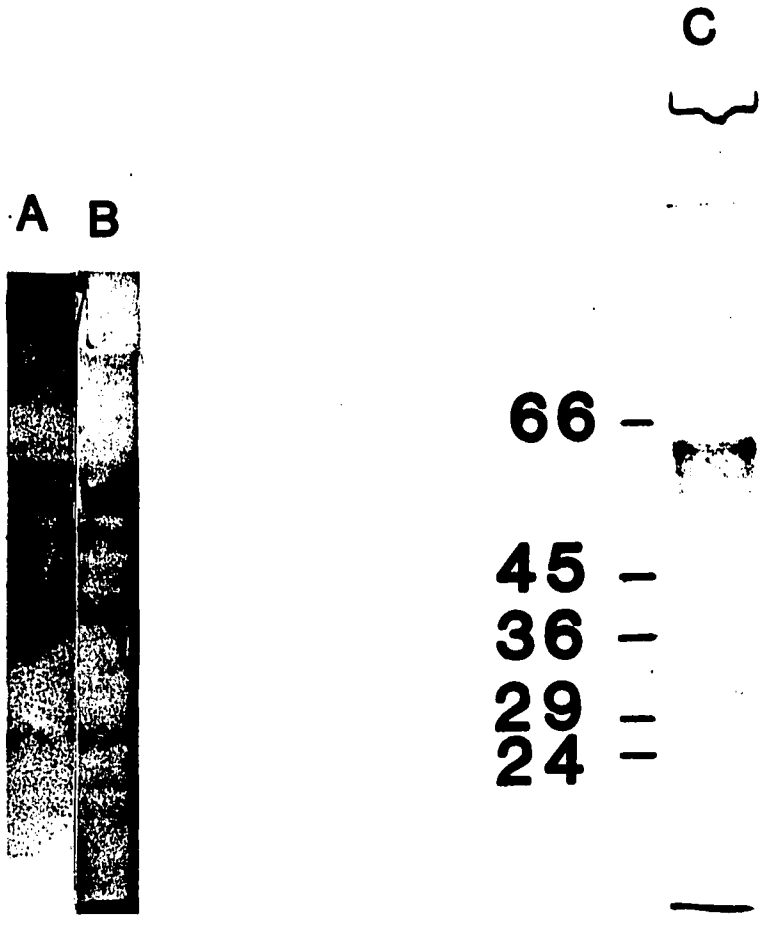

U/Fraction
0.0
0.1
0.2
0.3
0
10
20
30
Fraction #
Wash
0.2M KCl
2mM NADP

Table 1. Purification of Malic Enzyme

Step	Volume (ml)	Unit/ml[a]	Total U	Protein (mg/ml)	Total Protein (mg)	Specific Activity	Purification (fold)	Yield
Crude Mitochondria	22	0.43	9.5	5.37[b]	118	0.081	1	100%
30-70% $(NH_4)_2SO_4$	10	0.70	7.0	3.58[b]	36	0.20	2.4	73%
Sephadex	61	0.063	3.8	0.036[b]	2.2	1.7	21.6	40%
DEAE Cellulose	108	0.010	1.1	0.020[b]	2.2	5.1	62.8	11%
ATP Agarose	27	0.059	1.6	0.003[c]	0.081	18	222	16%

[a]One unit is defined as that amount of activity catalyzing the formation of one µmole of product per minute under conditions specified.
[b]Protein concentration determined by the method of Lowry.
[c]Protein concentration determined by the method of Bradford.

Figure 4. Native and SDS Polyacrylamide Gel Electrophoresis of the Purified Ribbed Mussel Gill ME. Lane A: native gel stained for ME activity (Total protein: 1 μg purified ME). Lane B: Native gel stained with silver (Total protein: 1 μg purified ME). Lane C: Denaturing gel stained with Coomassie (Total protein: 7 μg purified protein). Numbers and accompanying lines to the left of the photograph of the gel show the positions and molecular weights ($\times 10^{-3}$) of molecular weight standards.


C
A
B
66
45
36
29
24

the curves, apparent K_m's of 8.53 mM and 129 μM were found for pyruvate and NADPH, respectively. These values are higher than those obtained from estimating the K_m's from the curves (approximately 20 μM and 5 mM, for NADPH and pyruvate, respectively). Hyperbolic kinetics were observed with bicarbonate (Fig. 9) and the apparent K_m found for this substrate using Cleland's program was 19 mM. The enzyme therefore possessed relatively high affinities for malate and NADP, and relatively low affinities for bicarbonate, pyruvate, and NADPH.

NAD was found to produce only 4.5% of the enzyme activity observed with NADP (Fig. 10). However, when 1 mM NAD was added to assay mixtures containing various quantities of NADP, NAD was found to stimulate activity, not to act as an inhibitor. When an aliquot of enzyme preparation was concentrated 100 fold, electrophoresed under native conditions, and stained for malic enzyme activity under conditions described in Materials and Methods, two minor bands with relative mobilities corresponding to the mitochondrial MDH were observed. Therefore, this NAD dependent activity appeared to be due to MDH contamination.

The relative rates of the forward and reverse directions were measured under near optimal conditions. Specifically, a mixture of enzyme, 50 mM HEPES (pH 7), 10 mM malate, 156 μM NADP, and 1 mM $MnCl_2$ produced a reaction rate of 1.83 nmoles/minute was observed. With enzyme, 100 mM HEPES (pH 7), 20 mM pyruvate, 200 μM NADPH, 100 mM sodium bicarbonate, and 1 mM $MnCl_2$ a reaction rate of 0.91 nmoles/minute was

Figure 5. Kinetics of Malate Utilization by Ribbed Mussel Gill ME. Purified malic enzyme was added to a mixture of 50 mM HEPES (pH 7), 1 mM $MnCl_2$, 200 μM NADP, and the various concentrations of sodium malate indicated.

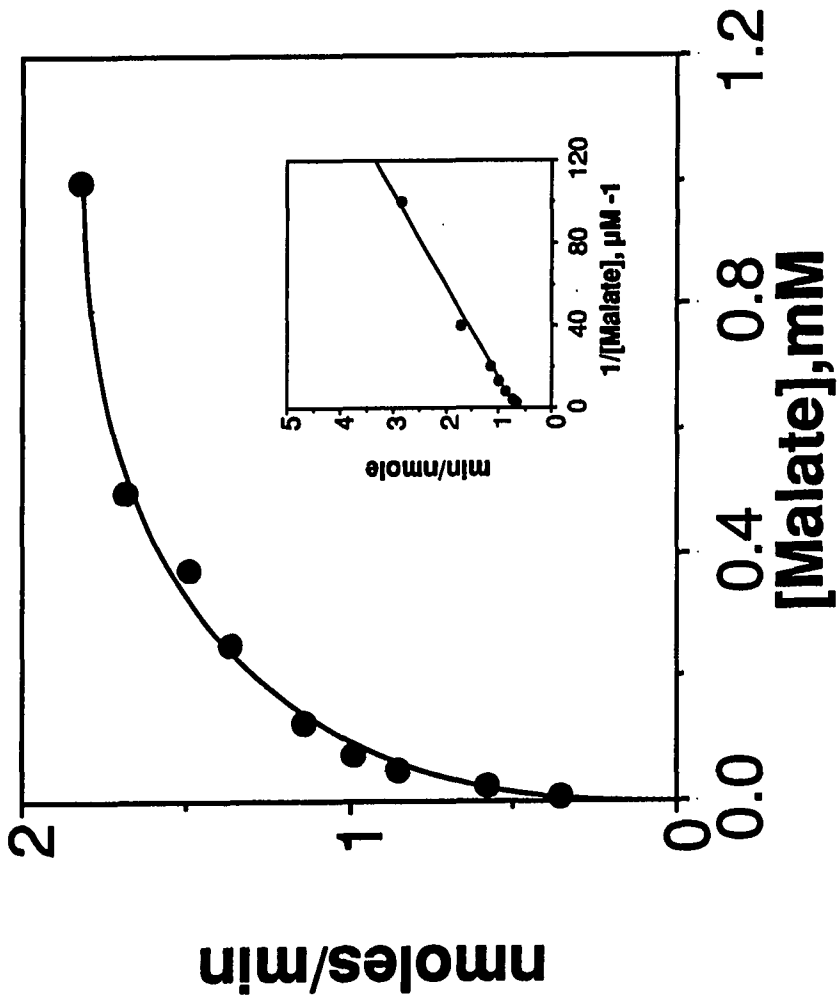

nmoles/min
2
1
0
0.0
0.4
0.8
1.2
[Malate],mM
min/nmole
5
4
3
2
1
0
0
40
80
120
1/[Malate], µM -1

Figure 6. Kinetics of NADP Utilization by the Ribbed Mussel Gill ME. Purifed malic enzyme was added to a mixture of 50 mM HEPES (pH 7), 1 mM $MnCl_2$, 10 mM malate, and the various concentrations of NADP indicated (closed circles). These assays were repeated with the various concentrations of NAD indicated (open circles).

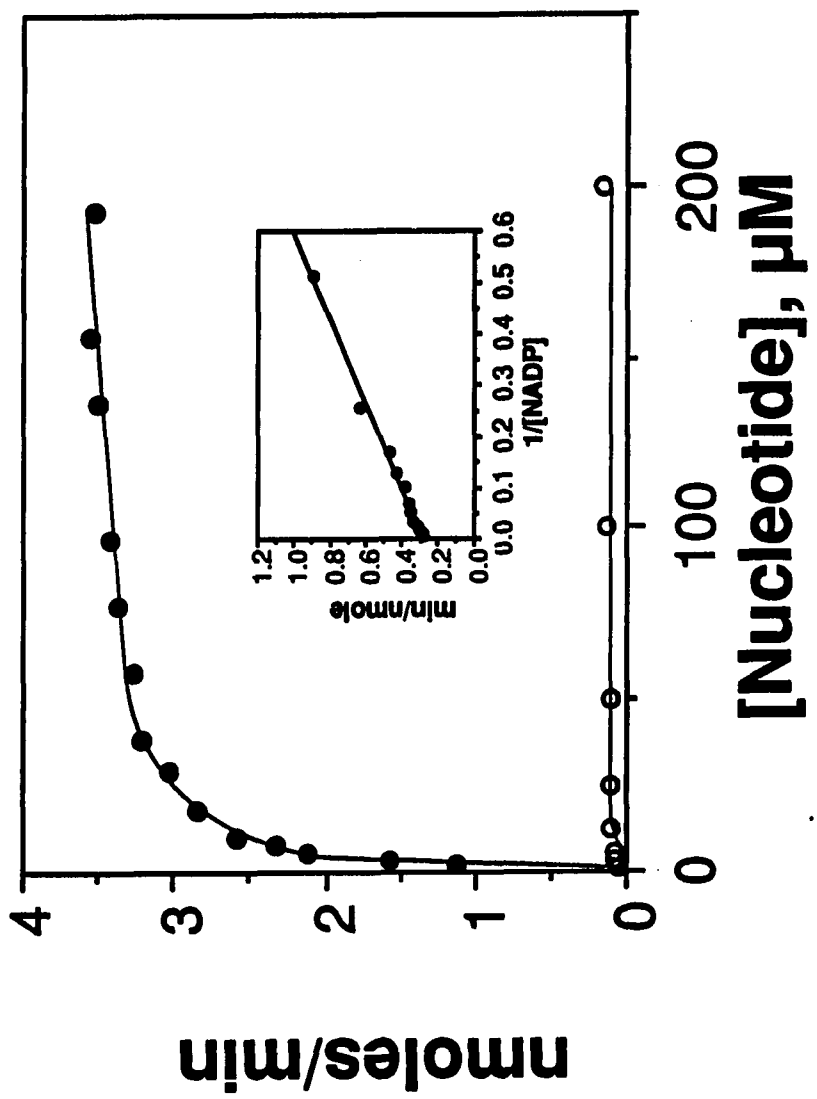

nmoles/min
4
3
2
1
0
0
100
200
[Nucleotide], µM
min/nmole
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0 0.1 0.2 0.3 0.4 0.5 0.6
1/[NADP]

Figure 7. Kinetics of Pyruvate Utilization by Ribbed Mussel Gill ME. Reaction mixtures contained purified enzyme, 50 mM HEPES (pH 7), 1 mM $MnCl_2$,100 mM sodium bicarbonate,200 μM NADPH and the pyruvate concentrations varied as indicated.

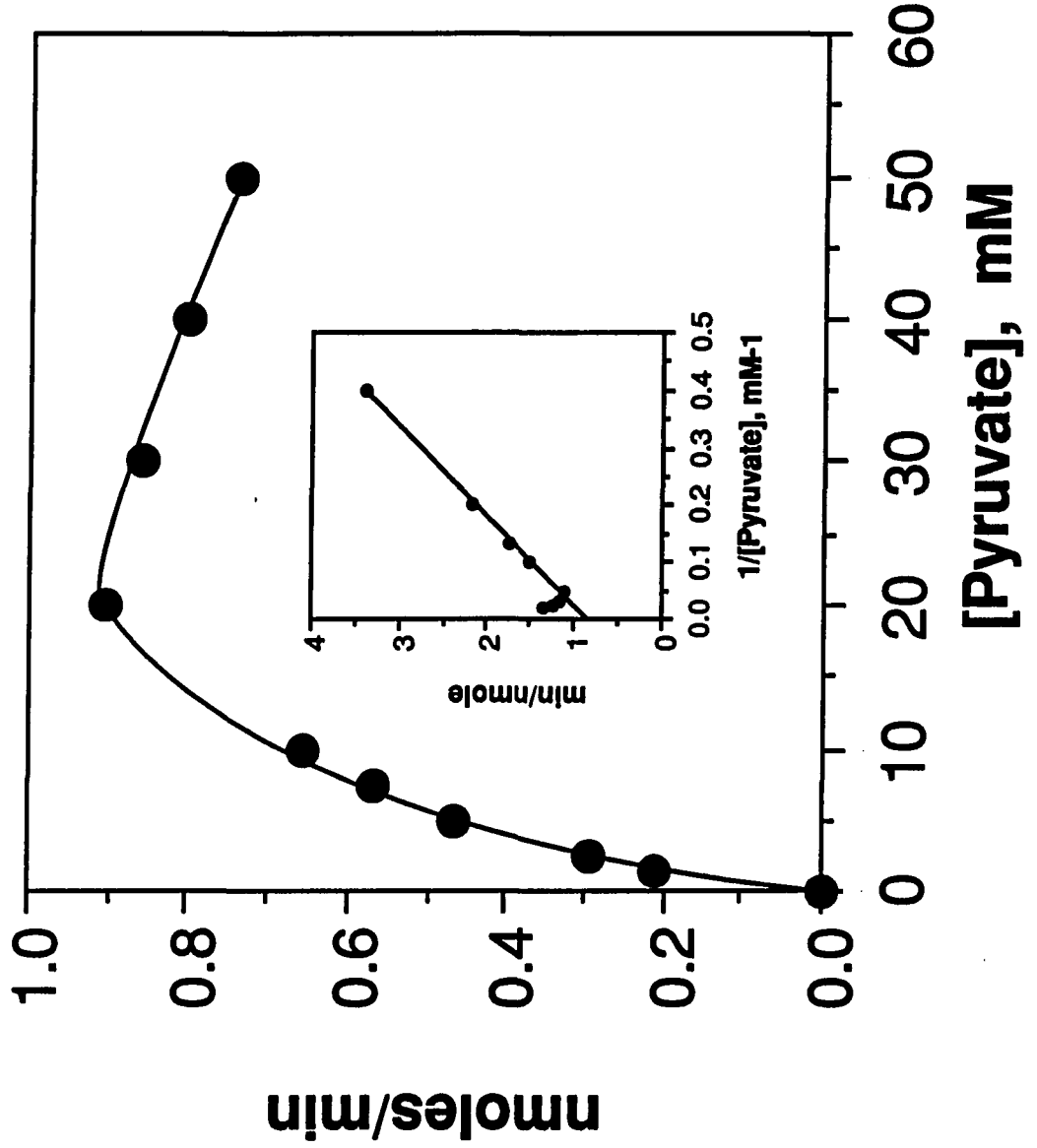

nmoles/min
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
50
60
[Pyruvate], mM
min/nmole
4
3
2
1
0
0.0 0.1 0.2 0.3 0.4 0.5
1/[Pyruvate], mM-1

Figure 8. Kinetics of NADPH Utilization by the Ribbed Mussel Gill ME. NADPH kinetics were determined in a mixtures containing purified enzyme, 100 mM HEPES (pH 7), 1 mM $MnCl_2$, 100 mM sodium bicarbonate, 50 mM pyruvate, and the indicated concentrations of NADPH.

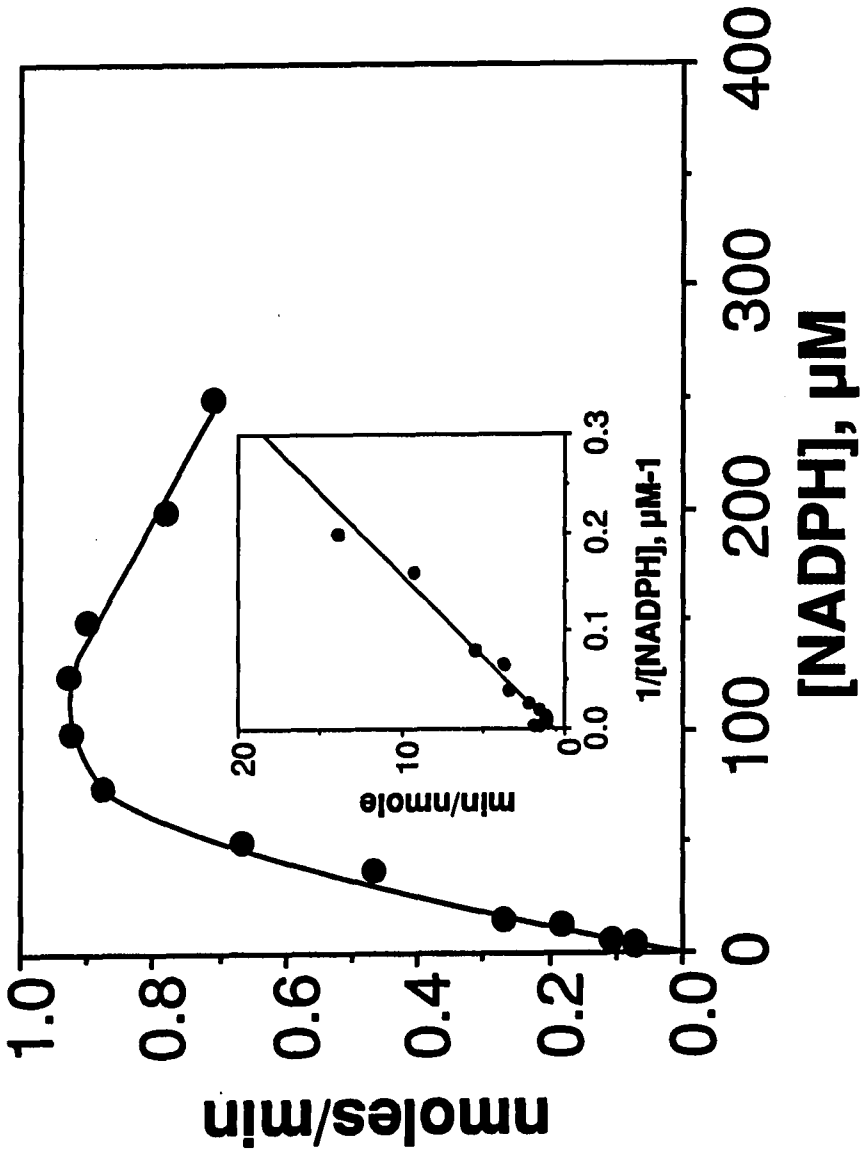

nmoles/min
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
[NADPH], µM
min/nmole
20
10
0
0.0
0.1
0.2
0.3
1/[NADPH], µM-1

Figure 9. Kinetics of Bicarbonate Utilization by the Ribbed Mussel Gill ME. The reaction mixtures contained purified enzyme, 100 mM HEPES (pH 7), 1 mM $MnCl_2$, 50 mM sodium pyruvate, 200 μM NADPH and the bicarbonate concentrations indicated.

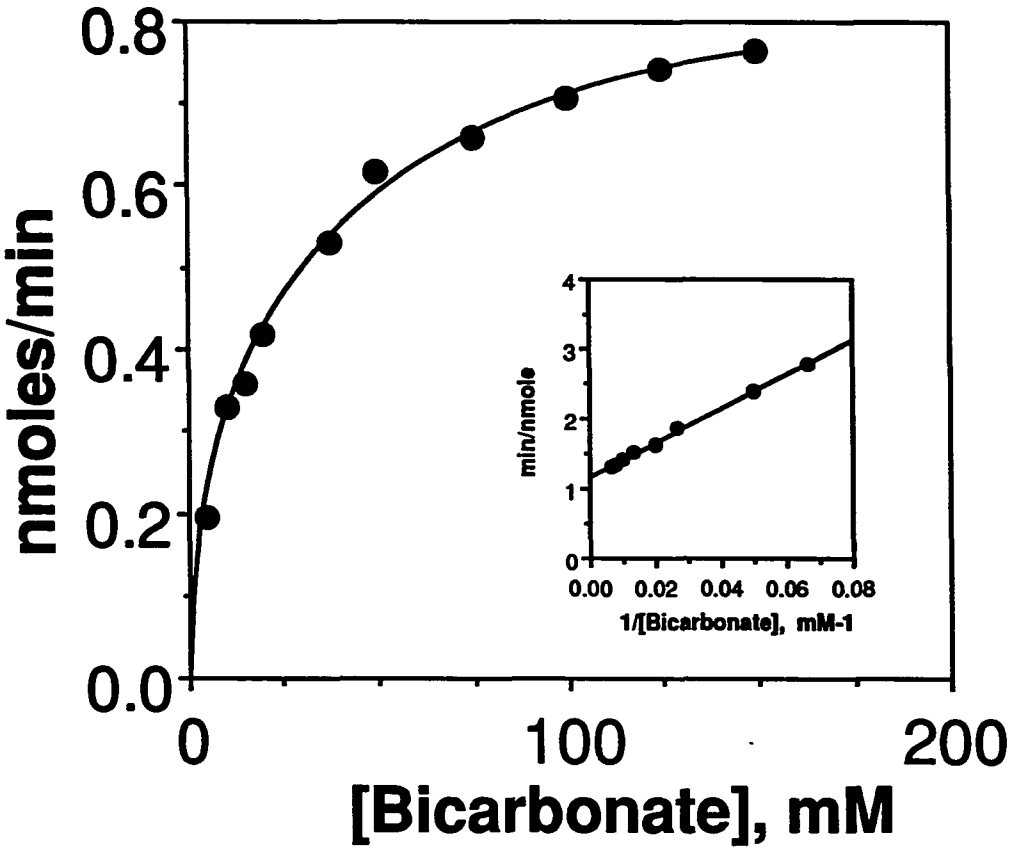
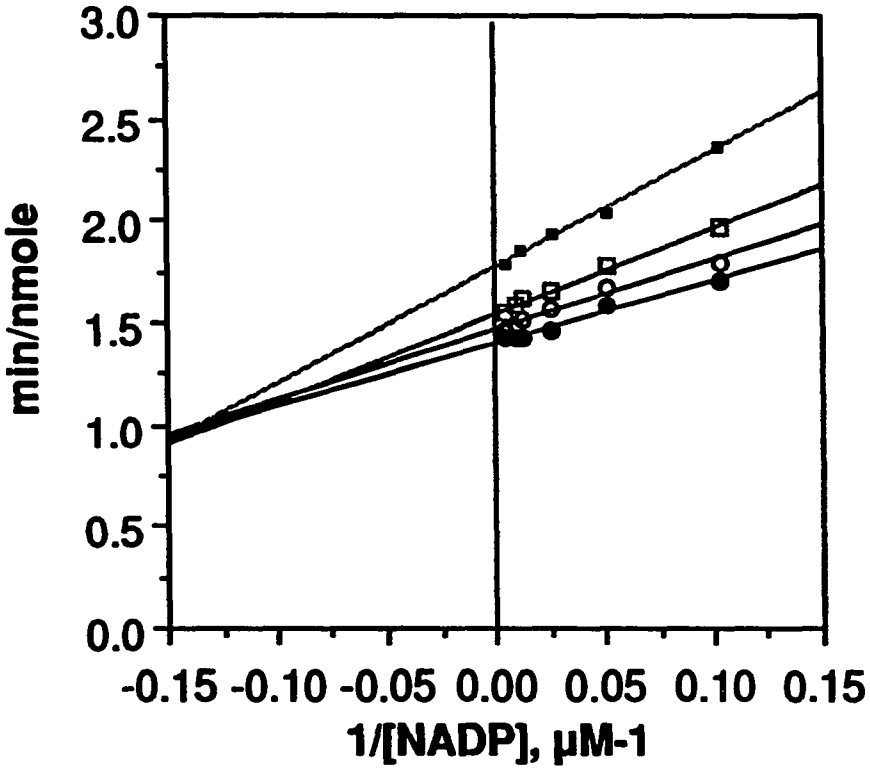
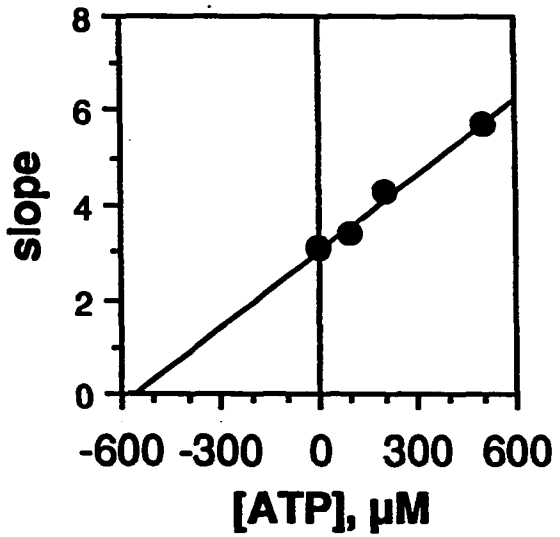

0.8
0.6
0.4
0.2
0.0
nmoles/min
0
100
200
[Bicarbonate], mM
4
3
2
1
0
min/nmole
0.00
0.02
0.04
0.06
0.08
1/[Bicarbonate], mM-1

Figure 10. ATP is a Mixed Inhibitor of NADP Utilization by the Ribbed Mussel Gill ME Catalyzed Reaction. Reaction mixtures contained 50 mM HEPES (pH 7), 10 mM malate, 1 mM $MnCl_2$, and the indicated concentrations of NADP and ATP. Closed circle: 0 μM ATP. Open circle: 100 μM ATP. Closed square: 200 μM ATP. Open square: 500 μM ATP.


3.0
2.5
2.0
1.5
1.0
0.5
0.0
min/nmole
-0.15 -0.10 -0.05 0.00 0.05 0.10 0.15
1/[NADP], µM-1


8
6
4
2
0
slope
-600 -300 0 300 600
[ATP], µM

observed. The forward reaction rate was therefore two times greater than the reverse rate at pH 7.

ATP was a mixed inhibitor of NADP (Fig. 10). The linear replots of slope as af function of ATP concentration indicated an apparent K_i of 0.51 mM for ATP.

DISCUSSION

As the native M_r =265,000 (Chapter 4), this mME appears to be tetrameric and composed of subunits of similar or identical molecular weight (Fig. 4). This mME is similar to MEs from many other animal tissues (Bagchi et al., 1986; Fodge et al., 1972; Hsu and Lardy, 1967; Nagel and Sauer, 1982; Saito et al., 1971). In mitochondria from many vertebrate tissues, a second smaller ME form (180,000) is sometimes observed in gel filtration studies (Lin and Davis, 1974; Sauer, 1973; Sauer et al., 1979; Taroni et al., 1988). Two forms with distinctive charge characteristics and nucleotide specificity have also been observed in the mitochondria of several herring tissues (Biegniewska et al., 1990). The molecular weights of bacterial MEs are quite variable. For example, the ME from the archaebacterium Solfonobus sulfataricus is a 105,000 MW dimer (Bartolucci et al., 1987), while the NADP-linked ME from E. coli is a 550,000 MW octomer (Spina et al., 1970). The NAD-linked ME from the potato is a 490,000 molecular weight octomer composed of two slightly different classes of subunits (Grover et al., 1981). The NADP-linked mME from the tapeworm Hymenolepis diminuta is unusual in that it appears to be a 120,000 molecular weight dimer (Li et al., 1972).

The specific activity of the purified enzyme (18 U/mg at pH 8, Table 1) is similar to the specific activities obtained for MEs from other sources. Specific activities between 27 and 40 are found for most vertebrate MEs (Moreadith and Lehninger, 1984; Hsu and Lardy, 1967; Saito et al, 1971). The specific activities of MEs from bacteria are considerably higher. Yamaguchi et al. (1973) report 177 U/mg for purified NAD-linked ME from E. coli whereas

Brown and Cook (1981) report 120 U/mg for the NADP-linked enzyme from this source.

This mME from ribbed mussel gill has only limited to use NAD as a cofactor, which is consistent with results previously reported for MEs from the sea mussel (deZwaan and van Marrewijk, 1973), the oyster (Hochachka and Mustafa, 1973) and preliminary results with partially purified mME from ribbed mussel gill (Chapter 4).

The apparent K_m's of the purified enzyme for its five substrates, malate, NADP, pyruvate, bicarbonate, and NADPH (Figs. 5-9), are comparable to those obtained for MEs from other sources (see Table 2). While malic enzyme reactivity with its substrates is influenced by pH, most studies included in the table were performed near physiological pH. Under the conditions that we used, the ME from ribbed mussel gill has a low apparent K_m for malate, being most similar to the ME from pigeon liver cytosol. Larger apparent K_m's for malate are observed for the MEs from the mitochondria of several invertebrates. However, all of the MEs listed in Table 2 have in common lower K_m's for malate than for pyruvate, and many including the ribbed mussel ME have lower K_m's for NADP than for NADPH. The substrate inhibition of the ribbed mussel ME by high concentrations of NADPH (Fig. 8) and pyruvate (Fig. 7) is an interesting characteristic of this enzyme. Substrate inhibition of this sort usually indicates that the reverse direction (i.e. malate formation) is not the physiologically preferred direction for this enzyme (Cornish-Bowden, 1979). The observed substrate inhibtion and the

Table 2: Kinetic Constants (Apparent K_m's) of Malic Enzymes from Various Sources[a]

Source	Malate	NADP	NAD	Pyruvate	Bicarbonate	NADPH	NADH	
Ribbed Mussel Mitochondria	43 µM	4 µM	X	8,000 µM	19,000 µM	129 µM	X	(Reported Here)
Sea Mussel Cytosol	1,050 µM	---	---	---	---	---	---	(deZwaan, 1977)
Mitochondria	1,050 µM	---	---	---	---	---	---	(deZwaan, 1977)
Squid Muscle	500 µM	10 µM	X	---	---	---	---	(Storey et al., 1975)
Oyster Adductor Cytosol	400 µM	20 µM	X	1,400 µM	---	---	---	(Hochachka and Mustafa, 1973)
Crayfish Abdomenal Muscle Mitochondria	660 µM	2.5 µM	X	---	---	---	---	(Skorkowski et al., 1977)
Ascaris sp. Mitochondria	1,900 µM	X	60 µM	5,600 µM	29,000 µM	X	60 µM	(Landsperger et al., 1978)
Leishmania donovani Promastigotes	180 µM	12 µM	X	200 µM	-----	40 µM	X	(Saadalla and Rassam, 1987)
Taenia crassiceps Cysticerci								
Mitochondria	410 µM	---	---	---	---	---	---	(Zenka and Prokopic, 1987)
Cytosol	130 µM	---	---	---	---	---	--	(Zenka and Prokopic, 1987)

Pig Heart Muscle								
Mitochondria	1,250 μM	1.9 μM	X	25,000 μM	---	---	---	(Bartholomé et al., 1972)
Cytosol	1,400 μM	1.2 μM	X	10,000 μM	---	---	---	(Bartholomé et al., 1972)
Human Skeletal Muscle								
Mitochondria	3,700 μM	780 μM	130 μM	---	---	---	---	(Taroni et al., 1988)
Pigeon liver								
Cytosol	86 μM	1.5 μM	X	6,400 μM	13,000 μM	2.1 μM	X	(Hsu et al., 1967)
Adrenal								
Mitochondria	2,500 μM	4.2 μM	X	5,000 μM	---	56 μM	X	(Simpson and Estabrook, 1969A)
Cytosol	540 μM	3.6 μM	X	2,300 μM	---	40 μM	X	(Simpson and Estabrook, 1969A)

[a]Where nucleotide specificity noted, value given under corresponding heading.
(X) denotes lack of ability for the enzyme to use a particular nucleotide.
(---) indicates that apparent K_m was not reported.

observation that the apparent K_m values obtained for pyruvate and NADP using Cleland's computer program are much higher than the values obtained by inspection of the curves may suggest that binding of these substrates is more complex than that explained by a hyperbolic model.

Whereas tetanic muscle contration seems to have little effect on ATP concentration (Gies, 1988), ATP concentration decreases in the adductor muscle of the closely related bivalve M. edulis in response to aerial exposure (Ebberink and deZwaan, 1980). This decrease in ATP concentration may influence the activities of several enzymes. ATP acts as a mixed inhibitor of NADP (Fig. 10) with a K_i of 0.51 mM. The ability of the nucleotide binding site to interact with ATP is also indicated by the observation that the enzyme adsorbs to an ATP affinity column in the presence of fumarate and $MnCl_2$. The failure of the enzyme to adsorb to the affinity column in the absence of fumarate and $MnCl_2$ may indicate a random reaction mechanism for this enzyme. Consistently, increased malate concentration increases the affinity of the enzyme for NADP, and pyruvate is a competitive inhibitor of malate. This feature is shared by the high activity NADP-linked ME from crustacean muscle mitochondria (Skorkowski et al., 1977). Interestingly, this other mME also fails to adsorb to an affinity column (2',5' ADP Sepharose) in the absence of added metal ion (Mn^{2+}) and dicarboxylic acid (malate) (Skorkowski and Storey, 1987). That succinate cannot substitute for fumarate in causing the ME activity from ribbed mussel gill tissue to stick to the ATP affinity column may be consistent with the observation that succinate does not compete as effectively as fumarate with malate binding to this ME.

Thermodynamic considerations predict that the oxidative decarboxylation of malate should be readily reversible as the free energy change is only 0.36 kcal/mole (Frenkel, 1972). Consistently, the rate of the forward reaction catalyzed by this ME is two times that of the reverse reaction when assayed in the presence optimzed concentrations of pyruvate, NADPH, and bicarbonate. However, several observations indicate that the reverse direction (ie reductive carboxylation) is not the favored direction, including the substrate inhibition exhibited at high NADPH and pyruvate concentrations, and the relatively low affinity of this activity for these substrates. As with other MEs, a major barrier to reductive carboxylation is the low affinity for bicarbonate. The apparent K_m's reported for bicarbonate range from 13 mM with the pigeon liver cME (Hsu et al., 1967) to 29 mM with the Ascaris suum mME (Landsperger et al., 1978). The K_m for the ribbed mussel ME is comparable with these other values (19 mM). Typical values for total carbonate concentrations in the hemolymph of estuarine bivalves range from 2 to 12 mM (Akberali and Trueman, 1985; Booth et al., 1984; Deaton, 1981; Dietz, 1979; Henry and Saintsing, 1983) indicating that the reverse reaction (malate formation) may not be feasible under ordinary conditions. The preferred reaction direction under ordinary conditions is therefore probably in the direction of pyruvate formation. However, the accumulation of acidic endproducts during prolonged anaerobic stress causes the release of Ca^{2+} and bicarbonate from shell dissolution with bicarbonate concentrations reaching 47 mM (see Burton, 1983). Therefore reversibility may be indicated during extreme anaerobiosis. The importance of the forward direction is of this enzyme by mitochondrial pyruvate metabolism. Pyruvate serves as substrate

for the synthesis of alanine by the alanine aminotransferase (Paynter et al., 1984a) localized within the mitochondria of ribbed mussel gill tissue. Pyruvate also serves as substrate for the active pyruvate dehydrogenase (Paynter et al., 1985b) also localized within the mitochondria of these animals. While pyruvate is metabolized within the mitochondria, it is apparently not transported there from the cytosol as indicated by the lack of stimulation by pyruvate of O_2 consumption by isolated bivalve mitochondria and also by the lack of pyruvate sparking of malate dependent O_2 consumption (Ballantyne and Moon, 1985; Burcham et al., 1984). Malate, however, serves as an excellent respiratory substrate for isolated bivalve mitochondria. The co-localization of this pyruvate forming ME activity with the alanine aminotransferase and the pyruvate dehydrogenase in the gill tissue from the ribbed mussel plus the apparent lack of pyruvate transport implicate malate as a precursor for the synthesis of alanine and acetate and the ME as an important activity in the conversion of malate into pyruvate.

SUMMARY AND CONCLUSIONS

To evaluate the role of malate metabolism in the accumulation of endproducts in environmentally stressed ribbed mussels, the cMDH and mMDH and the mME from the gill tissue were purified and subjected to detailed kinetic analyses. While cMDH has been purified previously from a gastropod (Lazou et al., 1987), this is the first report of purification of a malic enzyme or malate dehydrogenase from a mollusc to our knowledge.

The physical properties of the cMDH indicate that it is similar to cMDHs from diverse sources. It is a dimer with M_r= 60,000 composed of subunits with M_r= 32,000, as revealed by western blotting. The observation that it is immunologically similar to porcine cMDH indicates evolutionary conservation between these cMDHs.

The cMDH from the ribbed mussel gill is encoded by one genetic locus with two alleles present in the populations studied. MDH from the cytosol can be resolved into five electrophoretically distinct bands. The slow/slow (C1) and fast/fast (C3) homodimers and the slow/fast heterodimer (C2) are the least anodally migrating bands. Kinetic evidence suggests that the band designated C4 is probably a conformational variant of C3. Interestingly, C1, the least anodally migrating form, has the lowest apparent K_m for oxaloacetate, while forms C3-C4 have the highest apparent K_m for oxaloacete. The form with intermediate mobility (C2) has an apparent K_m for oxaloacetate intermediate between C1 and C3-C4. The form we designate C1 is the most prevalent in a ribbed mussel population studied. This may provide evidence

for selection at this locus. That the most anodally migrating form (C5) is probably of mitochondrial origin is indicated by kinetic characteristics more similar to the mitochondrial enzyme than the other cytosolic forms.

As intracellular, hemolymph, and mantle fluid pH drop in response to anaerobic exposure (Wijsman, 1975; Barrow et al., 1980; Ellington, 1983a; and Booth et al., 1980), the effect of pH on the reactivity of these enzymes with their substrate is of physiological relevance. For each of the cytosolic forms, variations in pH over the physiological range (pH 6-8) have little effect on the apparent K_m for oxaloacetate. The lack of a pH effect may indicate no enhancement of the cMDH redox reestablishing reaction during anaerobiosis. However, a detailed study of the effect of pH on the relative rates of the forward and reverse reactions of the C1 form indicates limited malate oxidation at the lower pH's observed during anaerobic exposure. This lack of reversibilty may favor oxaloacetate reduction.

When kinetics of the C1 isozyme are measured at pH 9.2 (an elevated pH chosen to permit the examination of the malate oxidizing reaction which is unfavorable at more physiological pH's), the apparent K_m's of oxaloacetate, NADH, and NAD are low (105, 17, and 38 μM, respectively) whereas the apparent K_m of malate is high (464 μM). This relatively low affinity for malate suggests that even at this non-physiological pH, malate formation is favored.

The cytosolic formation of malate from the reduction of oxaloacetate has also been proposed in parasitic helminths (Saz, 1981). Malate formed in the cytosol serves as a precursor for many compounds accumulating during

anaeroic stress in parasitic helminths as well as molluscs (deZwaan et al., 1981).

However, regulatory properties of cMDH suggest that aspartate may be a precursor for alanine or succinate formation which traverses the mitochondrial membrane as well. Increased NaCl concentration in the cell, such as might occur when the organism is subjected to hyperosmotic stress (Sarkissian and Gomolinski, 1976), strongly influences oxaloacetate binding to the cMDHs. Sodium chloride acts as a competitive inhibitor of the cMDHs. At constant oxaloacetate concentration, sodium chloride, potassium chloride, lithium chloride, and sodium acetate inhibit the C1 form approximately equally. Therefore, if increased salinity of the bathing medium increases the salt content of the tissues, the redox reestablishing oxaloacetate reduction may be inhibited during salt stress. This observation may explain the overall increase in reduction state of adenine dinucleotides during hyperosmotic stress (Baginski and Pierce, 1975, 1978). Inhibition of cMDH by succinate, and alanine may explain the overall increase in the reduction state of adenine dinucleotides during anaerobic stress (Baginski and Pierce, 1975, 1978). The inhibition of malate synthesis from oxaloacetate during hyperosmotic stress or anaerobic stress may favor aspartate synthesis in the cytosol by the cytosolic aspartate aminotranferase (Paynter et al., 1984b). Aspartate formed in the cytosol may then be transported into the mitochondria (Greenwalt, 1981) for alanine synthesis (Paynter et al., 1984a; Baginski and Pierce, 1977). Aspartate in the mitochondria is readily transaminated by the aspartate aminotransferase which favors oxaloacetate synthesis (Paynter et al., 1985b).

Because of the importance of malate and possibly oxaloacetate utilization in the mitochondria, the properties of the mMDH are of interest. The physical properties of the mMDH are similar to mMDHs from other sources. In its native state, it is a protein with M_r= 62,000 composed of subunits with M_r= 32,000. The mMDH is immunologically distinct from the cMDH, as it does not cross react with antibodies raised to porcine heart cMDH. It appears to have a slightly higher pH optimum for oxaloacetate reduction that the cytosolic form, and is more sensitive to substrate inhibition by millimolar concentrations of oxaloacetate than cMDH. The mMDH is also more sensitive to substrate inhibition by malate as the cMDH. When whole tissues are carefully prepared for electrophoresis in a buffer described by Klier (1989), only one mMDH band is observed. When mitochondria are separated and mMDHs are partially purified, a second less anodally migrating mMDH activity appears. Because these two forms when separated by DEAE cellulose chromatography have similar kinetic properties, it is likely that the less anodally migrating band may represent a conformational variant of the mMDH.

At pH 9.2, a pH chosen to overcome the unfavorable equilibrium observed at physiological pH's, the apparent K_m's for oxaloacetate, NADH, and NAD are low (47, 35, and 151 μM, respectively), whereas the apparent K_m for malate is high (1,060 μM). Also the substrate inhibition observed at high malate concentration may suggest limited malate utilization by this enzyme (Cornish-Bowden, 1979). Therefore, oxaloacetate utilization in the mitochondria is favored by the mMDH, as is observed for mMDHs from other sources and the cMDH from ribbed mussel. Malate production would

then be expected to supply carbon for the fumarase and fumarate reductase reactions. However, the appearance of radioactivity from succinate appears in aspartate and glutamate (Wijsman et al., 1977) during anaerobic incubation indicates that the mMDH is at least partially functioning in the oxaloacetate producing direction. Because of the kinetics favoring malate production, a supply of acetyl CoA must be made available for condensation with oxaloacetate (Skorkowski et al., 1984) to permit the forward operation of the enzyme. If PDH (Paynter et al., 1985b) shuts down under anaerobiosis, and the supply of acetyl CoA is limited, malate may be expected to accumulate. However, the anaerobic appearance of small quantities of acetate in the ribbed mussel (Ho and Zubkoff, 1983) indicates that the PDH reaction is at least partially active under anaerobic conditions. No anaerobic increase in malate is observed in tissues of the ribbed mussel (Ho and Zubkoff, 1982).

The responses of the cMDH and mMDH to several inhibitors differ. ATP is a competitive inhibitor of the cMDH, while it acts as a mixed non-competitive inhibitor of the mMDH. The cMDH apprears to be more sensitive to regulation by ATP (K_i = 0.77 mM) than the mMDH (K_i=5.38 mM). The mMDH is more sensitive to inhibition by the malate analogue hydroxymalonate than the cMDH. Hydroxymalonate acts as a competitive inhibitor of the mMDH (K_i=0.26 mM), but a mixed non-competitive inhibitor of the cMDH (K_i=1.17 mM). The mMDH is more sensitive to inhibition by sodium chloride, potassium chloride, and lithium chloride than the cMDH. The effect of increased salinity of the bathing medium on the osmotic composition of the mitochondria is not known. While sodium acetate inhibits the cMDH about equally as chloride derivatives, sodium acetate is a

inhibits the cMDH about equally as chloride derivatives, sodium acetate is a weaker inhibitor of mMDH than the chloride derivatives. Also, while alanine inhibits the cMDH, alanine has little effect on the mMDH. The relative lack of effect of alanine and acetate on the mMDH may permit the continuing operation of this enzyme under hyperosmotic or anaerobic conditions when alanine or alanine and acetate accumulate.

While carbon from malate supplied to isolated anaerobic mitochondria distributes mainly to succinate and propionate, a considerable amount appears in CO_2, acetate, and alanine as well (deZwaan et al., 1981). Tracer studies indicate that aspartate serves as alanine precursor during hyperosmotic stress (Harlocker et al., 1991) and anaerobic stress (Collicut and Hochachka, 1977). Because of the mitochondrial localization of the alanine aminotransferase in the gill of the ribbed mussel (Paynter et al., 1984a), mitochondrial decarboxylation of malate is indicated. Despite its presence in low total activity (Paynter et al., 1985b), the mitochondrial malic enzyme is likely to a major site of malate decarboxylation, and calculation of the rate of pyruvate formation by this enzyme indicates that this activity is more than sufficient to account for the appearance of alanine during hyperosmotic stress. In addition to the appearance of mitochondrially synthesized alanine, additional evidence for the importance of this reaction is indicated by a lack of pyruvate sparking of malate dependent O_2 consumption. Therefore, pyruvate needed for the PDH reaction, yielding acetyl CoA, must be synthesized in the mitochondria.

The physical properties of the malic enzyme from the ribbed mussel gill mME are similar to the MEs from a number of diverse sources (tetrameric with M_r=265,000). It has high affinity for metal ions. Low apparent K_m's are observed for malate, and NADP and while relatively high apparent K_m's are observed for pyruvate , NADPH, and bicarbonate. On the basis of thermodynamic considerations, the kinetics of the enzyme strongly favor pyruvate formation because of the limiting CO_2 concentration of the hemolymph and possibly tissues as well.

There are several characteristics which distinguish the ribbed mussel mitochondrial malic enzyme from other MEs previously described from other sources. It is not stimulated by dicarboxylic acids such as succinate and fumarate but is instead inhibited by them. However, the relative lack of inhibition by accumulating succinate as compared to fumarate indicates that succinate formation would not interfere with the continued formation of pyruvate and alanine under anaerobic conditions. It is relatively insensitive to CoA derivatives. While ATP inhibits NADP binding, it has no effect on malate binding.

The ribbed mussel mME appears to operate by a random reaction mechanism. Evidence for this reaction mechanism include: metal ion concentration influences malate binding, malate concentration influences NADP binding, pyruvate is a competitive inhibitor of malate, and the enzyme fails to adsorb to a ATP affinity column (which apparently interacts with the NADP binding site) when fumarate and $MnCl_2$ are omitted from the column buffer. The low apparent K_i of this enzyme for NADPH (16 μM)

indicates that this is probably the most potent regulator. Therefore when the state of reduction of mitochondrial nucleotides is increased, this malic enzyme may shut down. While pyruvate is a competitive inhibitor of malate, the apparent K_i is quite high (4.5 mM); therefore regulation by pyruvate is unlikely.

On the basis of the kinetic properties of the mMDH and the mME alone, a flow of carbon from oxaloacetate to malate into the accumulating endproducts is indicated. This observation along with the observation that the mitochondria possess an aspartate aminotransferase which favors aspartate degradation indicates that aspartate transported from the cytosol may serve as substrate for the formation of accumulating endproducts which can be sythesized from malate. The kinetic results are supported by analysis of total activities. mMDH strongly reduced oxaloacetate at pH 8, while the reverse reaction occurs only poorly at this pH. Reversal of carbon through the MDH to form malate should be exacerbated under conditions when concentration of acetyl CoA is limiting, such as with PDH shutdown caused by an increased NADH concentration during anaerobic or salt stress. The malate oxidizing activity possessed by the mME is also much greater than the malate oxidizing ability possessed by the mMDH.

The scheme suggested by these observations then is that malate is poorly oxidized by mMDH, but is largely directed toward either fumarase for fumarate and ultimately succinate and propionate formation, or mME for pyruvate formation. Alternatively, if aspartate is the form transported,

oxaloacetate formed from transamination is reduced to malate by the mMDH, which is then destined for succinate or pyruvate synthesis.

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